Patent
Attorney's Docket No. 021565-078

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

REQUEST FOR FILING CONTINUATION/DIVISIONAL APPLICATION UNDER 37 C.F.R. § 1.53(b)

Box PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a request for filing a [ ] continuation [X] divisional application under 37 C.F.R. § 1.53(b) of pending Application No. 09/176,320 filed on October 19, 1998, for RECOMBINANT PLANT EXPRESSING NON-COMPETITIVELY BINDING Bt INSECTICIDAL CRYSTAL PROTEINS, by the following named inventor(s):

(a) Full Name Herman VAN MELLAERT

(b) Full Name Johan BOTTERMAN

(c) Full Name Jeroen VAN RIE

(d) Full Name Henk JOOS

[X] The entire disclosure of the prior application from which a copy of the oath or declaration is supplied herewith is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

[ ] This application is being filed by less than all the inventors named in the prior application. In accordance with 37 C.F.R. 1.63(d)(2), the Commissioner is requested to delete the name(s) of the following person or persons who are not inventors of the invention being claimed in this application.

(a) Full Name _____

(b) Full Name _____

(c) Full Name _____

[ ] This application is being filed by more than all the inventors named in the prior application. In accordance with 37 C.F.R. 1.63(d)(2), the Commissioner is requested to add the name(s) of the following person or persons who are inventors of the invention being claimed in this application.

21839

(2/00)

(a) Full Name _____

(b) Full Name _____

(c) Full Name _____

1. [X] Enclosed is a copy of the prior Application No. 09/176,320 as originally filed on October 22, 1998, including copies of the specification, claims, drawings and the executed oath or declaration as filed.

2. [ ] Enclosed is a revised prior application and a copy of the prior executed oath or declaration as filed. No new matter has been added to the revised application.

3. [ ] _____ statement(s) claiming small entity status [ ] are enclosed [ ] were filed in prior Application No. _, filed on _.

4. [X] The filing fee is calculated below [X] and in accordance with the enclosed preliminary amendment:

CLAIMS					
	NO. OF CLAIMS		EXTRA CLAIMS	RATE	FEE
Basic Application Fee					$690.00 (101)
Total Claims	7	MINUS 20 =	---	x $18.00 (103) =	
Independent Claims	2	MINUS 3 =	---	x $78.00 (102) =	
If multiple dependent claims are presented, add $260.00 (104)					
Total Application Fee					$690.00
If small entity status is claimed, subtract 50% of Total Application Fee					
Add Assignment Recording Fee of if Assignment document is enclosed					
TOTAL APPLICATION FEE DUE					$690.00

5. [ ] Charge $ _____ to Deposit Account No. 02-4800 for the fee due.

6. [X] A check in the amount of $ 690.00 is enclosed for the fee due.

7. [X] The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800. This paper is submitted in duplicate.

8. [X] Cancel in this application original claims 2-19 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

9. [X] Amend the specification by inserting before the first line the sentence: --This application is a [ ] continuation, [X] divisional, of Application No. 09/176,320, filed October 22, 1998, which is a divisional of Application No. 08/465,609, filed June 5, 1995, which is a continuation of Application No. 08/173,274, filed December 23, 1993, which is a continuation of Application No. 07/640,400, filed January 22, 1991.--

10. [ ] Transfer the drawings from the pending prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate of this paper is enclosed for filing in the prior application file. (May only be used if signed by person authorized under 37 C.F.R. § 1.138 and before payment of issue fee.)

11. [X] New drawings are enclosed.

12. [X] Priority of Application No. 89401499.2 filed on May 31, 1989 in United Kingdom (country) and of PCT/EP90/00905 filed on May 30, 1990 is claimed under 35 U.S.C. § 119.

[X] The certified copy of the priority application

[ ] is enclosed

[X] was filed on _ in prior Application No. 07/640,400, filed on January 22, 1991

[ ] has not yet been filed.

13. [X] A preliminary amendment is enclosed.

14. [X] An Information Disclosure Statement is enclosed.

15. [ ] A General Authorization for Payment of Fees and Petitions for Extensions of Time is enclosed.

16. [X] Also enclosed Request for Transfer of Computer Sequence Listing with paper copy.

17. [X] The power of attorney in the prior application is to R. Danny Huntington.

a. [X] The power appears in the original papers in the prior application.

b. [ ] Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

c. [X] Recognize as Associate Attorney Malcolm K. McGowan, Ph.D., Reg. No. 39,300 .

d. [X] Address all future communications to: (May only be completed by applicant, or attorney or agent of record.)

R. Danny Huntington, Esq.
BURNS, DOANE, SWECKER & MATHIS, L.L.P.
P.O. Box 1404
Alexandria, Virginia 22313-1404

Date: September 13, 2000
Date

By: 
Malcolm K. McGowan, Ph.D.
Registration No. 39,300

ADDRESS OF
SIGNATOR:

BURNS, DOANE, SWECKER & MATHIS, L.L.P.
P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

[ ] inventor(s)
[ ] assignee of complete interest
[ ] attorney or agent of record
[X] filed under 37 C.F.R. § 1.34(a)

Patent
Attorney's Docket No. 021565-078

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

Herman VAN MELLAERT et al.

Application No.: TBA (Div of 09/176,320)

Filed: Even date herewith

For: RECOMBINANT PLANT EXPRESSING NON-COMPETITIVELY BINDING Bt INSECTICIDAL CRYSTAL PROTEINS

Group Art Unit: Unassigned

Examiner: Unassigned

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-cited Application on the merits, please amend the application as follows:

IN THE SPECIFICATION:

In compliance with 37 C.F.R. §1.823(a), please insert the attached paper copy of the "Sequence Listing" after the last page of the above-identified application (page 67).

On page 27, third line from the bottom, after "sequence" insert --[SEQ ID NO.: 1]--; and after "of" insert --[SEQ ID NO.: 2]--.

On page 29, line 17, after "sequence" insert --[SEQ ID NO.: 3]--;

line 25, after "sequence" insert --[SEQ ID NO.: 4]--.

IN THE CLAIMS:

Please delete claim 1 without prejudice to or disclaimer of the subject matter contained therein.

Please add the following new claims:

--20. An isolated protein comprising the amino acid sequence of the Bt14 protein, or an insecticidally effective fragment thereof.

21. An isolated protein comprising the amino acid sequence of the Bt15 protein, or an insecticidally effective fragment thereof.

22. An isolated DNA sequence encoding the protein or protein fragment of claim 20.

23. An isolated DNA sequence encoding the protein or protein fragment of claim 21.

24. A DNA molecule comprising the DNA of claim 22 operably linked to a promoter which can direct expression of said DNA in plant cells.

25. A DNA molecule comprising the DNA of claim 23 operably linked to a promoter which can direct expression of said DNA in plant cells.

26. A plant, seed, or plant cell comprising the DNA molecule of claim 24.

27. A plant, seed, or plant cell comprising the DNA molecule of claim 25.--

REMARKS

Entry of the foregoing and early and favorable consideration of the subject application is respectfully requested.

The paper copy of the Sequence Listing for the subject application, is by this amendment, added after the last page of the application (page 67).

By the present amendment, claim 1 has been deleted without prejudice to or disclaimer of the subject matter contained therein. New claims 20-27 are directed to Bt 14 and Bt15 proteins and insecticidal fragments thereof, DNA encoding those proteins and protein fragments, DNA constructs that include Bt14- and Bt15-encoding DNA, and plants, seeds, and plant cells comprising such DNA. These claims derive support from throughout the specification and claims as originally filed. No new matter has been added.

In the event that there are any questions concerning the present amendment, or the application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: _____
Malcolm K. McGowan, Ph.D.
Registration No. 39,300

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

Date: 13 September 2000

PREVENTION OF Bt RESISTANCE DEVELOPMENT

This invention relates to plant cells and plants, the genomes of which are transformed to contain at least two genes, each coding for a different non-competitively binding Bacillus thuringiensis ("B.thuringiensis" or "Bt") insecticidal crystal protein ("ICP") for a specific target insect species, preferably belonging to the order of Lepidoptera or Coleoptera. Such transformed plants have advantages over plants transformed with a single B. thuringiensis ICP gene, especially with respect to the prevention of resistance development in the target insect species against the at least two B. thuringiensis ICPs, expressed in such plants.

This invention also relates to a process for the production of such transgenic plants, taking into account the competitive and non-competitive binding properties of the at least two B. thuringiensis ICPs in the target insect species' midgut. Simultaneous expression in plants of the at least two genes, each coding for a different non-competitively binding B. thuringiensis ICP in plants, is particularly useful to prevent or delay resistance development of insects against the at least two B. thuringiensis ICPs expressed in the plants.

This invention further relates to a process for the construction of novel plant expression vectors and to the novel plant expression vectors themselves, which contain the at least two B. thuringiensis ICP genes encoding the at least two non-competitively binding B. thuringiensis ICPs. Such vectors allow integration and coordinate expression of the at least two B. thuringiensis ICP genes in plants.

BACKGROUND OF THE INVENTION

Since the development and the widespread use of chemical insecticides, the occurrence of resistant insect strains has been an important problem. Development of insecticide resistance is a phenomenon dependent on biochemical, physiological, genetic and ecological mechanisms. Currently, insect resistance has been reported against all major classes of chemical insecticides including chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroid compounds (Brattsten et al., 1986).

In contrast to the rapid development of insect resistance to synthetic insecticides, development of insect resistance to bacterial insecticides such as B. thuringiensis sprays has evolved slowly despite many years of use (Brattsten et al., 1986). The spore forming gram-positive bacterium B. thuringiensis produces a parasporal crystal which is composed of crystal proteins (ICPs) having insecticidal activity. Important factors decreasing the probability of emergence of resistant insect strains in the field against B. thuringiensis sprays are: firstly the short half-life of B. thuringiensis sprays after foliar application; secondly the fact that commercial B. thuringiensis preparations often consist of a mixture of several insecticidal factors including spores, ICPs and eventually beta-exotoxins (Shields, 1987); and thirdly the transitory nature of plant-pest interactions. Many successful field trials have shown that commercial preparations of a B. thuringiensis containing its spore-crystal complex, effectively control lepidopterous pests in agriculture and forestry (Krieg and Langenbruch, 1981). B. thuringiensis is at present the most widely used pathogen for microbial control of insect pests.

Various laboratory studies, in which selection against B. thuringiensis was applied over several generations of insects, have confirmed that resistance against B. thuringiensis is seldom obtained. However, it should be emphasized that the laboratory conditions represented rather low selection pressure conditions.

For example, Goldman et al. (1986) have applied selection with B. thuringiensis israelensis toxin over 14 generations of Aedes aegypti and found only a marginal decrease in sensitivity. The lack of any observable trend toward decreasing susceptibility in the selected strains may be a reflection of the low selection pressure (LC_{50}) carried out over a limited number of generations. However, it should be pointed out that Georghiou et al. (In : Insecticide Resistance in Mosquitoes : Research on new chemicals and techniques for management. In "Mosquito Control Research, Annual Report 1983, University of California.") with Culex quinquefasciatus obtained an 11-fold increase in resistance to B. thuringiensis israelensis after 32 generations at LC_{95} selection presssure.

McGaughey (1985) reported that the grain storage pest Plodia interpunctella developed resistance to the spore-crystal complex of B. thuringiensis; after 15 generations of selection with the Indian meal moth, Plodia interpunctella, using a commercial B. thuringiensis HD-1 preparation ("Dipel", Abbott Laboratories, North Chicago, Illinois 60064, USA), a 100-fold decrease in B. thuringiensis sensitivity was reported. Each of the colonies was cultured for several generations on a diet treated with a constant B. thuringiensis dosage which was expected to produce 70-90% larval mortality. Under these high selection presssure conditions, insect resistance to B.

thuringiensis increased rapidly. More recently, development of resistance against B. thuringiensis is also reported for the almond moth, Cadra cautella (McGaughey and Beeman, 1988). Resistance was stable when selection was discontinued and was inherited as a recessive trait (McGaughey and Beeman, 1988). The mechanism of insect resistance to B. thuringiensis toxins of Plodia interpunctella and Cadra cautella has not been elucidated.

The main cause of B. thuringiensis resistance development in both reported cases involving grain storage was the environmental conditions prevailing during the grain storage. Under the conditions in both cases, the environment was relatively stable, so B. thuringiensis degradation was slow and permitted successive generations of the pest to breed in the continuous presence of the microbial insecticide. The speed at which Plodia developed resistance to B. thuringiensis in one study suggests that it could do so within one single storage season in the bins of treated grain.

Although insect resistance development against B. thuringiensis has mostly been observed in laboratory and pilot scale studies, very recent indications of B. thuringiensis resistance development in Plutella xylostella populations in the (cabbage) field have been reported (Kirsch and Schmutterer, 1988). A number of factors have led to a continuous exposure of P. xylostella to B. thuringiensis in a relatively small geographic area. This and the short generation cycle of P. xylostella have seemingly led to an enormous selection pressure resulting in decreased susceptibility and increased resistance to B. thuringiensis.

A procedure for expressing a B. thuringiensis ICP gene in plants in order to render the plants insect-resistant (European patent publication ("EP") 0193259 [which is incorporated herein by reference]; Vaeck et al., 1987; Barton et al., 1987; Fischhoff et al., 1987) provides an entirely new approach to insect control in agriculture which is at the same time safe, environmentally attractive and cost-effective. An important determinant for the success of this approach will be whether insects will be able to develop resistance to B. thuringiensis ICPs expressed in transgenic plants (Vaeck et al., 1987; Barton et al., 1987; Fischhoff et al., 1987). In contrast with a foliar application, after which B. thuringiensis ICPs are rapidly degraded, the transgenic plants will exert a continuous selection pressure. It is clear from laboratory selection experiments that a continuous selection pressure has led to adaptation to B. thuringiensis and its components in several insect species. In this regard, it should be pointed out that the conditions in the laboratory which resulted in the development of insect-resistance to B. thuringiensis are very similar to the situation with transgenic plants which produce B. thuringiensis ICPs and provide a continuous selection pressure on insect populations feeding on the plants. Mathematical models of selection pressure predict that, if engineered insect-resistant plants become a permanent part of their environment, resistance development in insects will emerge rapidly (Gould, 1988). Thus, the chances for the development of insect resistance to B. thuringiensis in transgenic plants may be considerably increased as compared to the field application of B. thuringiensis sprays. A Heliothis virescens strain has been reported that is 20 times more resistant to B. thuringiensis HD-1 ICP

produced by transgenic Pseudomonas fluorescens and 6 times more resistant to the pure ICP (Stone et al., 1989). Furthermore, the monetary and human costs of resistance are difficult to assess, but loss of pesticide effectiveness invariably entails increased application frequencies and dosages and, finally, more expensive replacement compounds as new pesticides become more difficult to discover and develop.

Therefore, it would be desirable to develop means for delaying or even preventing the evolution of resistance to B. thuringiensis.

B. thuringiensis strains, active against Lepidoptera (Dulmage et al., 1981), Diptera (Goldberg and Margalit, 1977) and Coleoptera (Krieg et al., 1983), have been described. It has become clear that there is a substantial heterogeneity among ICPs from different strains active against Lepidoptera, as well as among ICPs from strains active against Coleoptera (Hofte and Whiteley, 1989). An overview of the different B. thuringiensis ICP genes, that have been characterized, is given in Table 2 (which follows the Examples herein).

Most of the anti-Lepidopteran B. thuringiensis (e.g., Bt3, Bt2, Bt73, Bt14, Bt15, Bt4, Bt18) ICP genes encode 130 to 140 kDa protoxins which dissolve in the alkaline environment of an insect's midgut and are proteolytically activated into an active toxin of 60-65 kDa. These ICPs are related and can be recognized as members of the same family based on sequence homologies. The sequence divergence however is substantial, and the insecticidal spectrum, among the order Lepidoptera, may be substantially different (Höfte et al., 1988).

The P2 toxin gene and the cry B2 gene are different from the above-mentioned genes in that they

do not encode high molecular weight protoxins but rather toxins of around 70 kDa (Donovan et al., 1988 and Widner and Whiteley, 1989, respectively).

It has recently become clear that heterogeneity exists also in the anti-Coleopteran toxin gene family. Whereas several previously reported toxin gene sequences from different B. thuringiensis isolates with anti-Coleopteran activity were identical (EP 0149162 and 0202739), the sequences and structure of bt21 and bt22 are substantially divergent (European patent application ("EPA") 89400428.2).

While the insecticidal spectra of B. thuringiensis ICPs are different, the major pathway of their toxic action is believed to be common. All B. thuringiensis ICPs, for which the mechanism of action has been studied in any detail, interact with the midgut epithelium of sensitive species and cause lysis of the epithelial cells (Knowles and Ellar, 1986) due to the fact that the permeability characteristics of the brush border membrane and the osmotic balance over this membrane are perturbed. In the pathway of toxic action of B. thuringiensis ICPs, the binding of the toxin to receptor sites on the brush border membrane of these cells is an important feature (Hofmann et al., 1988b). The toxin binding sites in the midgut can be regarded as an ICP-receptor since toxin is bound in a saturable way and with high affinity (Hofmann et al., 1988a).

Although this outline of the mode of action of B. thuringiensis ICPs is generally accepted, it remains a matter of discussion what the essential determinant(s) are for the differences in their insecticidal spectra. Haider et al. (1986) emphasize the importance of specific proteases in the insect midgut. Hofmann et al. (1988b) indicate that receptor binding is a prerequisite for toxic activity and describe that

Pieris brassicae has two distinct receptor populations for two toxins. Other authors have suggested that differences in the environment of the midgut (e.g., pH of the midgut) might be crucial.

SUMMARY OF THE INVENTION

In accordance with this invention, a plant is provided having, stably integrated into its genome, at least two B. thuringiensis ICP genes encoding at least two non-competitively binding insecticidal B. thuringiensis ICPs, preferably the active toxins thereof, against a specific target insect, preferably against a Lepidoptera or Coleoptera. Such a plant is characterized by the simultaneous expression of the at least two non-competitively binding B. thuringiensis ICPs.

Also in accordance with this invention, at least two ICP genes, particularly two genes or parts thereof coding for two non-competitively binding anti-Lepidopteran or anti-Coleopteran B. thuringiensis ICPs, are cloned into a plant expression vector. Plant cells transformed with this vector are characterized by the simultaneous expression of the at least two B. thuringiensis ICP genes. The resulting transformed plant cell can be used to produce a transformed plant in which the plant cells: 1. contain the at least two B. thuringiensis ICP genes or parts thereof encoding at least two non-competitively binding anti-Lepidopteran or anti-Coleopteran B. thuringiensis ICPs as a stable insert into their genome; and 2. express the genes simultaneously, thereby conferring on the plant improved resistance to at least one target species of insect, so as to prevent or delay development of resistance to B. thuringiensis of the at least one target species of insect feeding on the transformed plant.

Further in accordance with this invention, plant expression vectors are provided which allow integration and simultaneous expression of at least two B. thuringiensis ICP genes in a plant cell and which comprise one or more chimeric genes, each containing in the same transcriptional unit: a promoter which functions in the plant cell to direct the synthesis of mRNA encoded by one of the ICP genes; one or more different ICP genes, each encoding a non-competitively binding B. thuringiensis ICP; preferably a marker gene; a 3' non-translated DNA sequence which functions in the plant cell for 3' end formation and the addition of polyadenylate nucleotides to the 3'end of the mRNA; and optionally a DNA sequence encoding a protease-sensitive protein part between any two ICP genes.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, "B. thuringiensis ICP" (or "ICP") should be understood as an intact protein or a part thereof which has insecticidal activity and which can be produced in nature by B. thuringiensis. An ICP can be a protoxin, as well as an active toxin or another insecticidal truncated part of a protoxin which need not be crystalline and which need not be a naturally occurring protein. In this regard, an ICP can be a chimaeric toxin encoded by the combination of two variable regions of two different ICP genes as disclosed in EP 0228838.

As used herein, "protoxin" should be understood as the primary translation product of a full-length gene encoding an ICP.

As used herein, "toxin", "toxic core" or "active toxin" should all be understood as a part of a protoxin

which can be obtained by protease (e.g., by trypsin) cleavage and has insecticidal activity.

As used herein, "gene" should be understood as a full-length DNA sequence encoding a protein (e.g., such as is found in nature), as well as a truncated fragment thereof encoding at least the active part (i.e., toxin) of the protein encoded by the full-length DNA sequence, preferably encoding just the active part of the protein encoded by the full-length DNA sequence. A gene can be naturally occurring or synthetic.

As used herein, "truncated B. thuringiensis gene" should be understood as a fragment of a full-length B. thuringiensis gene which still encodes at least the toxic part of the B. thuringiensis ICP, preferentially the toxin.

As used herein, "marker gene" should be understood as a gene encoding a selectable marker (e.g., encoding antibiotic resistance) or a screenable marker (e.g., encoding a gene product which allows the quantitative analysis of transgenic plants).

Two ICPs are said to be "competitively binding ICPs" for a target insect species when one ICP competes for all ICP receptors of the other ICP, which receptors are present in the brush border membrane of the midgut of the target insect species.

Two ICPs are said to be "non-competitively binding ICPs" when, for at least one target insect species, the first ICP has at least one receptor for which the second ICP does not compete and the second ICP has at least one receptor for which the first ICP does not compete, which receptors are present in the brush border membrane of the midgut of the target insect species.

A "receptor" should be understood as a molecule, to which a ligand (here a B. thuringiensis ICP,

preferably a toxin) can bind with high affinity (typically a dissociation constant (Kd) between 10^{-11} and 10^{-6}M) and saturability. A determination of whether two ICPs are competitively or non-competitively binding ICPs can be made by determining whether: 1. a first ICP competes for all of the receptors of a second ICP when all the binding sites of the second ICP with an affinity in the range of about 10^{-11} to 10^{-6}M can be saturated with the first ICP in concentrations of the first ICP of about 10^{-5}M or less (e.g., down to about 10^{-11}M); and 2. the second ICP competes for the all of the receptors of the first ICP when all the binding sites of the first ICP with an affinity in the range of about 10^{-11} to 10^{-6}M can be saturated with the second ICP in concentrations of the second ICP of about 10^{-5}M or less.

General Procedures

This section describes in broad terms general procedures for the evaluation and exploitation of at least two B. thuringiensis ICP genes for prevention of the development, in a target insect, of a resistance to the B. thuringiensis ICPs expressed in transgenic plants of this invention. A non-exhaustive list of consecutive steps in the general procedure follows, after which are described particular Examples that are based on this methodology and that illustrate this invention.

In accordance with this invention, specific B. thuringiensis ICPs can be isolated in a conventional manner from the respective strains such as are listed in Table 2 (which follows the Examples). The ICPs can be used to prepare monoclonal or polyclonal antibodies specific for these ICPs in a conventional manner (Höfte et al., 1988).

The ICP genes can each be isolated from their respective strains in a conventional manner. Preferably, the ICP genes are each identified by: digesting total DNA from their respective strains with suitable restriction enzyme(s); size fractionating the DNA fragments, so produced, into DNA fractions of 5 to 10 Kb; ligating such fractions to suitable cloning vectors (e.g., pEcoR251, deposited at the Deutsche Sammlung von Mikroorganismen und Zellculturen ("DSM"), Braunschweig, Federal Republic of Germany, under accession number no. 4711 on July 13, 1988); transforming E.coli with the cloning vectors; and screening the clones with a suitable DNA probe. The DNA probe can be constructed from a highly conserved region which is commonly present in different B. thuringiensis genes which encode crystal protoxins against Coleoptera or Lepidoptera, such as on the basis of an N-terminal amino acid sequence determined by gas-phase sequencing of the purified proteins (EPA 88402115.5).

Alternatively, the desired fragments, prepared from total DNA of the respective strains, can be ligated in suitable expression vectors (e.g., a pUC vector (Yanisch-Perron et al., 1985) with the insert under the control of the lac promoter) and transformed in E. coli, and the clones can then be screened by conventional colony immunoprobing methods (French et al., 1986) for expression of the toxins with monoclonal or polyclonal antibodies raised against the toxins produced by the strains.

The isolated B. thuringiensis ICP genes can then be sequenced in a conventional manner using well-known procedures (e.g., Maxam and Gilbert, 1980).

At present, several ICP genes have been cloned from different subspecies of B. thuringiensis (Table 2). The nucleotide sequences from several of these B.

thuringiensis ICP genes have been reported. Whereas several sequences are identical or nearly identical and represent the same gene or slight variants of the same gene, several sequences display substantial heterogeneity and show the existence of different B. thuringiensis ICP gene classes. Several lines of evidence suggest that all these genes specify a family of related insecticidal proteins. Analysis of the distribution of B. thuringiensis ICPs in different B. thuringiensis strains by determining the protein composition of their crystals, by immunodetection using polyclonal antisera or monoclonals against purified crystals, or by using gene-specific probes, shows that subspecies of B. thuringiensis might contain up to three related B. thuringiensis ICP genes belonging to different classes (Kronstad et al., 1983).

To express the isolated and characterized gene in a heterologous host for purification and characterization of the recombinant protein, the preferred organism is Escherichia coli. A number of expression vectors for enhanced expression of heterologous genes in E. coli have been described (e.g., Remaut et al., 1981). Usually the gene is cloned under control of a strong regulatable promoter, such as the lambda pL or pR promoters (e.g., Botterman and Zabeau, 1987), the lac promoter (e.g., Fuller, 1982) or the tac promoter (e.g., De Boer et al., 1983), and provided with suitable translation initiation sites (e.g., Stanssens et al, 1985 and 1987). Gene cassettes of the B. thuringiensis ICP genes can be generated by site-directed mutagenesis, for example according to the procedure described by Stanssens et al. (1985 and 1987). This allows cassettes to be made comprising, for example, a truncated ICP gene fragment encoding the toxic core (i.e., toxin) of an ICP or a hybrid gene

encoding the toxic core and a selectable marker according to the procedures described in EPA 88402241.9.

The cells of an E. coli culture, which has been induced to produce a recombinant ICP, are harvested. The method used to induce the cells to produce the recombinant ICP depends on the choice of the promoter. For example, the lac promoter (Fuller, 1982) is induced by isopropyl-B-D-thiogalacto-pyranoside ("IPTG"); the pL promoter is induced by temperature shock (Bernard et al., 1979). The recombinant ICP is usually deposited in the cells as insoluble inclusions (Hsuing and Becker, 1988). The cells are lysed to liberate the inclusions. The bulk of E. coli proteins is removed in subsequent washing steps. A semi-purified protoxin pellet is obtained, from which the protoxin can be dissolved in alkaline buffer (e.g., Na_2CO_3, pH 10). The procedure for the ICP Bt2, which is also applicable to other recombinant toxins, has been described by Höfte et al., 1986.

In accordance with this invention, the binding of various ICPs to ICP receptors on the brush border membrane of the columnar midgut epithelial cells of various insect species has been investigated. The brush border membrane is the primary target of each ICP, and membrane vesicles, preferentially derived from the brush border membrane, can be obtained according to Wolfersberger et al., 1987.

The binding to ICP receptors of one or more ICPs (e.g., ICP A, ICP B, etc.) can be characterized by the following steps (Hofmann et al, 1988b):

1. ICP A is labelled with a suitable marker (usually a radioisotope such as ^{125}I).
2. Brush border membranes are incubated with a small amount (preferably less than 10^{-10} M) of labelled

ICP A together with different concentrations of non-labelled ICP A (preferably from less than 10^{-11} to 10^{-5} M).

3. For all concentrations tested the amount of labelled ICP A bound to the brush border membranes is measured.
4. Mathematical analysis of these data allows one to calculate various characteristics of the ICP receptor such as the magnitude of the population of binding sites (Scatchard, 1949).
5. Competition by other toxins (e.g. ICP B) is preferably studied by incubating the same amount of labelled ICP A with brush border membranes in combination with different amounts of ICP B (preferentially from 10^{-11} to 10^{-6} M; and subsequently, steps 3 and 4 are repeated.

By this procedure, it has been found, for example, that Bt3 toxin, Bt2 toxin and Bt73 toxin are competitively binding anti-Lepidopteran ICPs for Manduca sexta and Heliothis virescens (See example 6 which follows). Various other combinations of toxins have been found to be non-competitively binding anti-Lepidopteran or anti-Coleopteran toxins (example 6).

Although the concept of competitivity versus non-competitivity of ICP binding does not have any practical importance by itself, the observation of the non-competitivity of two B. thuringiensis ICPs, active against the same target insect, can be put to very significant practical use. This is because a combination of two non-competitively binding B. thuringiensis ICPs can be used to prevent development, by a target insect, of resistance against such B. thuringienis ICPs.

A selection experiment with M. sexta, using Bt2 toxin, Bt18 toxin, and a mixture of Bt2 and Bt18

toxins, has shown that Bt2 and Bt18 are two non-competitively binding anti-Lepidopteran toxins. After 20 generations of selection, a very pronounced reduction in ICP sensitivity was observed in the selection experiments with Bt2 or Bt18 alone (>100 times). The reduction in sensitivity in the selection experiment with a Bt2-Bt18 mixture was only marginal (3 times). This demonstrates the unexpected practical advantage of a simultaneous use of two non-competitively binding ICPs in a situation which models the high selection pressure which will exist with the use of transgenic plants transformed with ICP genes. In this regard, the two resistant strains showed a specific loss in receptor sites for either the Bt2 or Bt18 toxin. In each case, receptor sites for the toxin, which was not used for selection, were not affected or their concentration even increased. Thus, the Bt2 selected strain retained its Bt18 receptors, and the Bt18 selected strain developed an increased number of Bt2 receptors. Indeed, the Bt18 selected strain showed an increased sensitivity for Bt2 along with its increased Bt2 receptor concentration. No significant changes in receptor sites were found in the strain selected against the combined toxins. These findings are described in detail in Example 7 which follows.

A similar mechanism of resistance to Bt has been observed with respect to a strain of diamondback moth, Plutella xylostella. This strain had developed resistance in the field to Dipel which is a commercial formulation of the Bt HD-1 strain. Crystals of Dipel comprise a mixture of several BtICPs, similar to the Bt2, Bt3 and Bt73 proteins which are competitively-binding ICPs. As shown by both insect bioassays and competitive binding studies using Bt2 and Bt15, the Dipel-resistant diamondback moth strain is resistant to

Bt2 protoxin and toxin but maintains full sensitivity to Bt15 protoxin and toxin. This finding is relevant to other combinations of non-competitively binding anti-Lepidopteran or Coleopteran ICPs which are expected to have the same beneficial effect against their common target insects.

Hence, a combination of non-competitively binding ICPs, when directly expressed in a transgenic plant, offers the substantial advantage of reducing the chances of development of insect resistance against the ICPs expressed in the plant. There may be additional benefits because the combined spectrum of two toxins may be broader than the spectrum of a single ICP expressed in a plant (See Examples 8, 9 and 10 which follow).

If, among two competitively binding ICPs, one has a larger binding site population than the other against a given target insect, it will be most advantageous to use the one with the larger population of binding sites to control the target pest in combination with the most suitable non-competitively binding B. thuringiensis ICP. For example, as seen from Example 6, it is preferred to use Bt73 against Heliothis virescens, rather than Bt2 or Bt3, and it is preferred to use Bt3 against Manduca sexta rather than Bt2 or Bt73. The selected gene can then be combined with the best suitable non-competitively binding ICP.

Previously, plant transformations involved the introduction of a marker gene together with a single ICP gene, within the same plasmid, in the plant genome (e.g., Vaeck et al., 1987; Fischoff et al., 1987). Such chimeric ICP genes usually comprised either all or part of an ICP gene, preferably a truncated ICP gene fragment encoding the toxic core, fused to a selectable marker gene, such as the neo gene coding for neomycin

phosphotransferase. The chimeric ICP gene was placed between the T-DNA border repeats for Agrobacterium Ti-plasmid mediated transformation (EP 0193259).

This invention involves the combined expression of two or even more B. thuringiensis ICP genes in transgenic plants. The insecticidally effective B. thuringiensis ICP genes, encoding two non-competitively binding ICPs for a target insect species, preferably encoding the respective truncated ICP genes, are inserted in a plant cell genome, preferably in its nuclear genome, so that the inserted genes are downstream of, and under the control of, a promoter which can direct the expression of the genes in the plant cell. This is preferably accomplished by inserting, in the plant cell genome, one or more chimaeric genes, each containing in the same transcriptional unit: at least one ICP gene; preferably a marker gene; and optionally a DNA sequence encoding a protease (e.g., trypsin)-sensitive or -cleavable protein part intercalated in frame between any two ICP genes in the chimaeric gene. Each chimaeric gene also contains at least one promoter which can direct expression of its ICP gene in the plant cell.

The selection of suitable promoters for the chimaeric genes of this invention is not critical. Preferred promoters for such chimaeric genes include: the strong constitutive 35S promoter obtained from the cauliflower mosaic virus, isolates CM 1841 (Gardner et al., 1981), CabbB-S (Franck et al., 1980) and CabbB-JI (Hull and Howell, 1987); the promoter of the nopaline synthetase gene ("PNOS") of the Ti-plasmid (Herrera-Estrella, 1983); the promoter of the octopine synthase gene ("POCS" [De Greve et al., 1982]); and the wound-inducible TR1' promoter and the TR2' promoter which drive the expression of the 1' and 2' genes,

respectively, of the T-DNA (Velten et al., 1984). Alternatively, a promoter can be utilized which is specific for one or more tissues or organs of the plant, whereby the inserted genes are expressed only in cells of the specific tissue(s) or organ(s). Examples of such promoters are a stem-specific promoter such as the AdoMet-synthetase promoter (Peleman et al., 1989), a tuber-specific promoter (Rocha-Sosa et al., 1989), and a seed-specific promoter such as the 2S promoter (Krebbers et al., 1988). The ICP genes could also be selectively expressed in the leaves of a plant (e.g., potato) by placing the genes under the control of a light-inducible promoter such as the promoter of the ribulose-1,5-bisphosphate carboxylase small subunit gene of the plant itself or of another plant such as pea as disclosed in EP 0193259. Another alternative is to use a promoter whose expression is inducible (e.g., by temperature or chemical factors).

A 3' non-translated DNA sequence, which functions in plant cells for 3' end formation and the polyadenylation of the 3' end of the mRNA sequence encoded by the at least one ICP gene in the plant cell, also forms part of each such chimeric gene. The selection of a suitable 3' non-translated DNA sequence is not critical. Examples are the 3' untranslated end of the octopine synthase gene, the nopaline synthase gene or the T-DNA gene 7 (Velten and Schell, 1985).

The selection of marker genes for the chimaeric genes of this invention also is not critical, and any conventional DNA sequence can be used which encodes a protein or polypeptide which renders plant cells, expressing the DNA sequence, readily distinguishable from plant cells not expressing the DNA sequence (EP 0344029). The marker gene can be under the control of its own promoter and have its own 3' non-translated DNA

sequence as disclosed above, provided the marker gene is in the same genetic locus as the ICP gene(s) which it identifies. The marker gene can be, for example: a herbicide resistance gene such as the sfr or sfrv genes (EPA 87400141); a gene encoding a modified target enzyme for a herbicide having a lower affinity for the herbicide than the natural (non-modified) target enzyme, such as a modified 5-EPSP as a target for glyphosate (U.S. patent 4,535,060; EP 0218571) or a modified glutamine synthetase as a target for a glutamine synthetase inhibitor (EP 0240972); or an antibiotic resistance gene, such as a neo gene (PCT publication WO 84/02913; EP 0193259).

Using A. tumefaciens Ti vector-mediated plant transformation methodology, all chimeric genes of this invention can be inserted into plant cell genomes after the chimaeric genes have been placed between the T-DNA border repeats of suitable disarmed Ti-plasmid vectors (Deblaere et al., 1988). This transformation can be carried out in a conventional manner, for example as described in EP 0116718, PCT publication WO 84/02913 and EPA 87400544.0. The chimeric genes can also be in non-specific plasmid vectors which can be used for direct gene transfer (e.g., as described by Pazkowski et al., 1984; De La Pena et al., 1986). Different conventional procedures can be followed to obtain a combined expression of two B.thuringiensis ICP genes in transgenic plants as summarized below.

I Chimeric gene constructs whereby two or more ICP genes and a marker gene are transferred to the plant genome as a single piece of DNA and lead to the insertion in a single locus in the genome

Ia The genes can be engineered in different transcriptional units each under control of a distinct promoter

To express two or more ICP genes and a marker gene as separate transcriptional units, several promoter fragments directing expression in plant cells can be used as described above. All combinations of the promoters mentioned above in the chimaeric constructs for one ICP gene are possible. Examples of such individual chimeric constructs are described for the bt2 gene in EP 0193259, for the bt13 gene in EPA 88402115.5 and for the bt18 gene in EPA 88402241.9. The ICP gene in each chimeric gene of this invention can be the intact ICP gene or preferably an insecticidally-effective part of the intact ICP gene, especially a truncated gene fragment encoding the toxic core of the ICP. The individual chimeric genes are cloned in the same plasmid vector according to standard procedures (e.g., EP 0193259).

Ib Two genes (e.g., either an ICP and a marker gene or two ICP genes) or more can be combined in the same transcriptional unit

To express two or more ICP genes in the same transcriptional unit, the following cases can be distinguished:

In a first case, hybrid genes in which the coding region of one gene is in frame fused with the coding region of another gene can be placed under the control of a single promoter. Fusions can be made between either an ICP and a marker gene or between two ICP genes. An example of an ICP gene-marker gene fusion has been described in EP 0193259 (i.e., a hybrid truncated bt2-neo gene encoding a Bt2 toxin-NPTII fusion protein).

Another possibility is the fusion of two ICP genes. Between each gene encoding an ICP which still is insecticidally active (i.e., a toxic part of the protoxin), a gene fragment encoding a protease (e.g.,

trypsin) - sensitive protein part should be included, such as a gene fragment encoding a part of the N-terminal or C-terminal amino acid sequence of one of the ICPs which is removed or cleaved upon activation by the midgut enzymes of the target insect species.

In a second case, the coding regions of the two respective ICP genes can be combined in dicistronic units placed under the control of a promoter. The coding regions of the two ICP genes are placed after each other with an intergenic sequence of defined length. A single messenger RNA molecule is generated, leading to the translation into two separate gene products. Based on a modified scanning model (Kozak, 1987), the concept of reinitiation of translation has been accepted provided that a termination codon in frame with the upstream ATG precedes the downstream ATG. Experimental data also demonstrated that the plant translational machinery is able to synthesize several polypeptides from a polycistronic mRNA (Angenon et al., 1989).

II Chimeric constructs with one or more ICP genes that are transferred to the genome of a plant already transformed with a one or more ICP genes

Several genes can be introduced into a plant cell during sequential transformation steps (retransformation), provided that an alternative system to select transformants is available for the second round of transformation. This retransformation leads to the combined expression of ICP genes which are introduced at multiple loci in the genome. Preferably, two different selectable marker genes are used in the two consecutive transformation steps. The first marker is used for selection of transformed cells in the first transformation, while the second marker is used for selection of transformants in the second round of

transformation. Sequential transformation steps using kanamycin and hygromycin have been described, for example by Sandler et al. (1988) and Delauney et al. (1988).

III Chimeric constructs with one or more ICP genes, that are separately transferred to the nuclear genome of separate plants in independent transformation events and are subsequently combined in a single plant genome through crosses.

The first plant should be a plant transformed with a first ICP gene or an F1 plant derived herefrom through selfing (preferably an F1 plant which is homozygous for the ICP gene). The second plant should be a plant transformed with a second ICP gene or an F1 plant derived herefrom through selfing (preferably an F1 plant which is homozygous for the second ICP gene). Selection methods can be applied to the plants obtained from this cross in order to select those plants having the two ICP genes present in their genome (e.g., Southern blotting) and expressing the two ICPs (e.g., separate ELISA detection of the immunologically different ICPs). This is a useful strategy to produce hybrid varieties from two parental lines, each transformed with a different ICP gene, as well as to produce inbred lines containing two different ICP genes through crossing of two independent transformants (or their F1 selfed offspring) from the same inbred line.

IV Chimeric constructs with one or more ICP genes separately transferred to the genome of a single plant in the same transformation experiment leading to the insertion of the respective chimeric genes at multiple loci.

Cotransformation involves the simultaneous transformation of a plant with two different expression vectors, one containing a first ICP gene, the second

containing a second ICP gene. Along with each ICP gene, a different marker gene can be used, and selection can be made with the two markers simultaneously. Alternatively, a single marker can be used, and a sufficiently large number of selected plants can be screened in order to find those plants having the two ICP genes (e.g., by Southern blotting) and expressing the two proteins (e.g., by means of ELISA). Cotransformation with more than one T-DNA can be accomplished by using simultaneously two different strains of Agrobacterium, each with a different Ti-plasmid (Depicker et al., 1985) or with one strain of Agrobacterium containing two T-DNAs on separate plasmids (de Framond et al., 1986). Direct gene transfer, using a mixture of two plasmids, can also be employed to cotransform plant cells with a selectable and a non-selectable gene (Schocher et al., 1986).

The transgenic plant obtained can be used in further plant breeding schemes. The transformed plant can be selfed to obtain a plant which is homozygous for the inserted genes. If the plant is an inbred line, this homozygous plant can be used to produce seeds directly or as a parental line for a hybrid variety. The gene can also be crossed into open pollinated populations or other inbred lines of the same plant using conventional plant breeding approaches.

Of course other plant transformation methods can be used and are within the scope of the invention as long as they result is a plant which expresses two or more non-competitively binding ICPs. In this regard, this invention is not limited to the use of Agrobacterium Ti-plasmids for transforming plant cells with genes encoding non-competitively binding ICPs. Other known methods for plant cell transformations, such as electroporation or by the use of a vector

system based on plant viruses or pollen, can be used for transforming monocotyledonous and dicotyledonous plants in order to obtain plants which express two non-competitively binding ICPs. Furthermore, DNA sequences encoding two non-competitively binding ICPs other than those disclosed herein can be used for transforming plants. Also, each of the ICP genes, described herein, can be encoded by equivalent DNA sequences, taking into consideration the degeneracy of the genetic code. Also, equivalent ICPs with only a few amino acids changed, such as would be obtained through mutations in the ICP gene, can also be used, provided they encode a protein with essentially the same characteristics (e.g., insecticidal activity and receptor binding).

The following Examples illustrate the invention. Those skilled in the art will, however, recognize that other combinations of two or more non-competitively binding B. thuringiensis ICP genes can be used to transform plants in accordance with this invention in order to prevent the development, in a target insect, of resistance to B. thuringiensis ICPs expressed in the transformed plants. Unless otherwise indicated, all procedures for making and manipulating DNA were carried out by the standardized procedures described in Maniatis et al, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982).

EXAMPLE 1: Collection of genes

The collection of anti-Lepidopteran and anti-Coleopteran Bt genes encoding ICPs, which are the subject of the Examples, is described in Table 2 (following the Examples). References for the respective genes are indicated in Table 2. The origin, the isolation and characterization of the Bt genes, which have not been published, are described below. Bt

strains, such as strains HD-1, HD-68, HD-110, and HD-73, are publicly available from the Agricultural Research Culture Collection, Northern Regional Research Laboratory, U.S. Dept. of Agriculture, Peoria, Illinois 61604, U.S.A.

bt3

gene: From B. thuringiensis var. kurstaki HD-1, the ICP was cloned. Characterization of this gene revealed an open reading frame of 3528 bp which encodes a protoxin of 133 kDa. This gene was identical to the one described by Schnepf et al. (1985).

bt73

gene: From B. thuringiensis var HD-73. The ICP gene was cloned as described by Adang et al. (1985).

bt4

gene: A genomic library was prepared from total DNA of strain B. thuringiensis aizawai HD-68. Using the 1.1 kb internal HindIII fragment of the bt2 gene as a probe, a gene designated bt4 was isolated. Characterization of this gene revealed an open reading frame of 3495 bp which encodes a protoxin of 132 kDa and a trypsin activated toxin fragment of 60 kDa. This (insect controlling protein) gene differs from previously identified genes and was also found in several other strains of subspecies aizawai and entomocidus including HD-110. Fig. 13 shows the nucleotide sequence and deduced amino acid sequence of the open reading frame ("ORF") of the bt4 gene extending from nucleotide 264 to nucleotide 3761.

bt14 and bt15

genes: A genomic library was prepared from total DNA of strain B. thuringiensis var. entomocidus HD-110 by partial Sau3A digest of the total DNA and cloning in the vector pEcoR251 (deposited at DSM under

accession number 4711). Using monoclonal antibodies (Höfte et al., 1988), at least three structurally distinct ICPs were identified in crystals of B. thuringiensis entomocidus HD-110. These monoclonal antibodies were used to clone the three different ICP genes from this B. thuringiensis strain. One of these genes is the bt4 gene as described above.

The second gene was called "bt15". Fig. 14 shows the nucleotide sequence and deduced amino acid sequence of the ORF of the bt15 gene, isolated from HD-110, extending from nucleotide 234 to nucleotide 3803. The Shine and Dalgarno sequence, preceding the initiation codon is underlined. This gene has an open reading frame of 3567 bp which encodes a protoxin of 135 kDa and a 63 kDa toxin fragment. A similar gene has been described by Honee et al. 1988, isolated from B. thuringiensis entomocidus 60.5. The bt15 gene differs from the published sequence at three positions: an Ala codon (GCA) is present instead of an Arg codon (CGA) at position 925 and a consecution of a Thr-His codon (ACGCAT) is present instead of a Thr-Asp codon (ACCGAT) at position 1400. (The numbers of the positions are according to Honnee et al., 1988). Another similar gene has been described in EP 0295156, isolated from B. thuringiensis aizawai 7-29 and entomocidus 6-01. The bt15 gene is different from this published nucleotide sequence at three different places : 1) a Glu codon (GAA) instead of an Ala codon (GCA) at position 700; 2) the sequence TGG, CCA, GCG, CCA instead of TGC, CAG, CGC, CAC, CAT at position 1456 and 3) an Arg codon (CGT) instead of an Ala codon (GCG) at

position 2654. (The numbers of the positions are according to EP 0295156).

The third gene isolated was called "bt14". It has an open reading frame of 3621 bp which encodes a 137 kDa protoxin and a 66 kDa activated toxin fragment. A similar gene has been cloned from B.thuringiensis HD-2 (Brizzard and Whiteley, 1988). The bt14 gene differs from the published nucleotide sequence by two nucleotide substitutions: a T instead of a C at position 126, and a C instead of a T at position 448 (the numbers of the positions are according to Brizzard and Whiteley, 1988). In the first case, the Ile codon (ATT or ATC) is conserved whereas in the second case the Tyr codon (TAT) is converted to a His codon (CAC).

bt2

gene: The bt2 gene was cloned as described in EP 0193259.

bt18

gene: Cloning of the bt18 gene was performed as described in EPA 88402241.9.

bt13

gene: The bt13 gene was cloned as described in EPA 88402115.5.

bt21 and bt22

genes: These genes, encoding Coleopteran-active ICPs, were cloned as described in EPA 89400428.2.

EXAMPLE 2 : Construction of gene cassettes and expression of Bt genes in E.coli

1) bt2, bt18: the construction of bt2 and bt18 gene cassettes has been previously described in EPA 86300291.1 and 88402241.9, respectively. Basically, they comprise a truncated gene encoding the toxic core and a hybrid gene comprising the

truncated gene fused in frame to the N-terminus of the neo gene. The gene cassettes are used to transform E. coli to express the Bt2 and Bt18 ICP toxins.

2) bt14, bt15: as described in EPA 88402241.9, gene cassettes for the bt14 and bt15 genes were constructed in order to express the genes in E.coli and in plants.

First, a NcoI site was introduced at the N-terminus of the genes by site-directed mutagenesis.

In the case of the bt15 gene, the conversion of the TT nucleotides, immediately in front of the ATG codon, into CC yielded a NcoI site overlapping with the ATG initiation codon. This site was introduced using the pMa/c vectors for site-directed mutagenesis (Stanssens et al., 1987) and a 28-mer oligonucleotide with the following sequence:

5'-CGGAGGTATTCCATGGAGGAAAATAATC-3'.

This yielded the plasmid pVE29 carrying the N-terminal fragment of the bt15 gene with a NcoI site at the ATG initiation codon.

According to Brizzard and Whiteley (1988), the initiation codon of the bt14 gene is a TTG codon. Thus, a NcoI site was created in a like manner at this codon for site directed mutagenesis using a 34-mer oligonucleotide with the following sequence :

5'-CCTATTTGAAGCCATGGTAACTCCTCCTTTTATG-3'.

In this case the sequence of the intitiation codon was converted from ATATTGA to ACCATGG. This yielded the plasmid pHW44 carrying the N-terminal fragment of the bt14 gene with a NcoI site at the initiation codon.

In a second step, the genes were reconstructed by ligating the N-terminal gene fragments with a suitable C-terminal gene fragment, yielding a bt15 gene and bt14 gene with a NcoI site at the ATG initiation codon.

To express the bt14 and bt15 genes encoding the protoxin in E. coli, the following constructs were made: pOH50 containing the bt15 gene under the control of the lac promoter; and pHW67 containing the bt14 gene under the control of the tac promoter. Induction of a culture of the E. coli strain WK6 carrying the respective plasmids with IPTG yielded an overproduced protein (Fuller, 1982).

The active toxic fragments of the Bt15 and Bt14 protoxins comprise 63 and 60 kDa trypsin digest products respectively. Instead of expressing the whole bt15 or bt14 gene, it is also possible to express a toxin-encoding gene fragment or derivative thereof in plants. To this end, truncated bt14 and bt15 gene fragments were constructed. In order to be able to select transgenic plants producing the ICP gene products, hybrid genes of the truncated gene fragments were also made with the neo gene encoding a selectable marker as described in EP 0193259.

By comparison of the nucleotide sequence of the bt4, bt14 and bt15 genes, respectively, with the bt2 and bt18 genes, respectively, the BclI site could be identified as a suitable site localized downstream of the coding sequence encoding the toxin gene fragment. To construct a truncated gene fragment and a hybrid gene of the truncated gene fragment with the neo gene, the filled BclI site was ligated to the filled EcoRI site of pLKM91 (Höfte et al., 1986) and the filled HindIII site of pLK94 respectively (Botterman and Zabeau, 1987). pLKM91 carries a 5' truncated neo gene fragment which codes for an enzymatically active C-terminal gene fragment of the neo gene, and pLK94 contains translation stop codons in three reading frames. This yielded the following plasmids which are then used to transform E. coli to express the ICP

genes: pHW71 carrying a truncated bt14-neo hybrid gene; pHW72 carrying a truncated bt14 gene; pVE34 carrying a truncated bt15-neo hybrid gene; and pVE35 carrying a truncated bt15 gene.

In a similar way as described for the bt14 and bt15 genes, gene cassettes are constructed for the bt3 and bt4 genes which are then expressed in E.coli.

EXAMPLE 3: Purification of recombinant ICPs

The ICPs expressed in E. coli in Example 2 are purified by the method (described for recombinant Bt2 protoxin) by Höfte et al. (1986).

EXAMPLE 4: Purification of toxins

Solubilized protoxins of Bt2, Bt3, Bt73, Bt4, Bt14, Bt15, Bt18, Bt13, Bt21 and Bt22 (in Na_2CO_3 50mM, DTT 10 mM pH=10) are dialyzed against 0.5 % $(NH_4)_2CO_3$ at pH 8 and treated with trypsin (trypsin/protoxin=1/20 w/w) for 2h at 37°C. The activated toxin is chromatographically purified (Mono-Q column on FPLC) as described by Hofmann et al.(1988b).

EXAMPLE 5: Determination of the insecticidal spectrum

The ICP protoxins and toxins of Examples 3 and 4 are evaluated for their insecticidal activity. Each protoxin is dissolved in alkaline buffer containing a reducing agent (Na_2CO_3 50 mM, DTT 10 mM pH=10), and each toxin is used as soluble protein directly from FPLC. Protein concentrations are determined. Subsequently, dilutions of the resulting protoxin or toxin solution are prepared in PBS buffer pH=7.4 containing 0.15 M NaCl and 0.1 % bovine serum albumin ("BSA").

The artificial medium for insect culture, described by Bell and Joachim (1976) for Manduca sexta, is poured in appropriate receptacles and allowed to solidify. Subsequently a quantity of the (pro)toxin dilutions is applied on this medium, and the water is

allowed to evaporate under a laminar flow. This results in a medium with a certain quantity (in the range of 0.1 to 10000 ng/cm2) of toxin coated on its surface. For example, for the Bt2 toxin, typical dilutions for a toxicity test on Manduca sexta are 1, 5, 25, 125 and 625 ng/cm2. First instar larvae of Manduca sexta are then applied on the coated medium, and growth and mortality are assessed after 6 days. Mortality increases with dosage. Dose response data is analysed in probit analysis (Finney, 1962), and the data are best summarized by an LD_{50} value which is the amount of toxin which kills 50 % of the insects. The LD_{50} for Bt2 toxin against Manduca sexta is around 20 ng/cm2.

Similar assays are carried out for other insect species using a suitable diet or by applying the ICPs on leaves for insects, for which no artificial diet is used.

EXAMPLE 6: Binding studies

Toxins

All protoxins and their toxic fragments were purified according to the methods described for the Bt2 protoxin and toxin in Höfte et al. (1986) and EP 0193259. The activated and purified toxins are further referred to as the Bt2, Bt3, Bt73, Bt4, Bt14, Bt15, Bt18, Bt13, Bt21 and Bt22 toxins.

By way of example for the Bt73 toxin, it has been shown that B. thuringiensis var. kurstaki HD73 produces a protein of 133 kDa encoded by a 6.6 kb type gene. A culture of this strain was grown as described by Mahillon and Delcour (1984). The autolysed culture was spun down (20 minutes at 4500 rpm in a HB4 rotor) and washed with a buffer containing 20 mM Tris, 100 mM NaCl and 0.05 % Triton X-100, pH 8. The final pellet was resuspended in this buffer (4 ml buffer for 100 ml culture). This solution was then layered onto a linear

Urograffin gradient (60-70%) which was centrifuged in a SW 28 rotor for 90 minutes at 18000 rpm. Crystals were collected and stored at -20° C until further use. Activation was performed according to Höfte et al. (1986). The purified toxin is further referred to as the Bt73 toxin.

Iodination of ICPs

Iodination of Bt2, Bt3, and Bt73 toxins was performed using the Chloramin-T method (Hunter and Greenwood, 1962). 1 mCi ^{125}I-NaI and 20 to 37.5 ug Chloramin-T in NaCl/P_i were added to 50 ug of purified toxin. After gentle shaking for 60 seconds, the reaction was stopped by adding 53 ug of potassium metabisulfite in H_2O. The whole mixture was loaded on a PD 10 Sephadex G-25M gelfiltration column to remove free iodine. A subsequent run on a Biogel P-60 column was carried out in order to increase the purity.

Alternatively, toxins were labeled using the Iodogen method. Iodogen (Pierce) was dissolved in chloroform at 0.1 mg/ml. 100 ul of this solution was pipetted into a disposable glass vessel and dried under a stream of nitrogen gas. The vessel was rinsed with Tris buffer (20 mM Tris, pH 8.65 with 0.15 M NaCl). 50 ug of toxin (in Tris buffer) was incubated with 1 mCi of ^{125}I-NaI in the tube for 10 minutes. The reaction was then stopped by the addition of 1 M NaI (one fourth of the sample volume). The sample was immediately loaded onto a PD10 Sephadex G-25M column and later on a Biogel P-60 column to remove free iodine and possible degradation products.

Other toxins were iodinated using one of the above mentioned procedures.

Determination of specific activity of iodinated toxin

Specific activity of iodinated Bt2, Bt3, and Bt73 toxin samples was determined using a "sandwich" ELISA

technique according to Voller, Bidwell and Barlett (1976). Primary antibody was a polyclonal antiserum raised against Bt2 toxin, and the secondary antibody was a monoclonal antibody 4D6.

The conjugate used was alkaline phosphatase coupled to anti-mouse IgG. The reaction intensity of a standard dilution series of unlabeled toxin and dilutions of the iodinated toxin sample (in NaCl/P_i - 0.1 % BSA) was measured. Linear regression calculations yielded the protein content of the radioactive toxin sample. The samples with the highest specific activities were used in the binding assays. Specific activities were 59400, 33000 and 19800 Ci/mole (on reference date) for Bt73 toxin (labeled according to Iodogen procedure), Bt2 toxin (Chloramin-T method) and Bt3 toxin (Iodogen method) respectively.

Specific activities of other toxins were determined using a similar approach. Specific monoclonal and polyclonal antibodies for each of these toxins were raised and applied in ELISA.

Preparation of brush border membrane vesicles

Brush border membrane vesicles ("BBMV") from Manduca sexta, Heliothis virescens, Plutella xylostella, Phthorimaea operculella, Spodoptera exigua, Spodoptera littoralis, Plodia interpunctella, Mamestra brassicae, Pieris brassicae and Leptinotarsa decemlineata were prepared according to the method of Wolfersberger et al. (1987). This is a differential centrifugation method that makes use of the higher density of negative electrostatic charges on luminal than on basolateral membranes to separate these fractions.

Binding assay

Duplicate samples of ^{125}I-labeled toxin, either alone or in combination with varying amounts of

unlabeled toxin, were incubated at the appropriate temperature with brush border membrane vesicles in a total volume of 100 ul of Tris buffer (Tris 10 mM, 150 mM NaCl, pH 7.4). All buffers contained 0.1 % BSA. The incubation temperature was 20 C. Ultrafiltration through Whatman GF/F glass fiber filters was used to separate bound from free toxin. Each filter was rapidly washed with 5 ml of ice-cold buffer ($NaCl/P_i$- 0.1 % BSA). The radioactivity of the filter was measured in a gammacounter (1275 Minigamma, LKB). Binding data were analyzed using the LIGAND computer program. This program calculates the bound concentration of ligand as a function of the total concentration of ligand, given the affinity (Ka or its inverse Kd = 1/Ka, the dissociation constant) and the total concentration of receptors or binding site concentration (R_t).

Determination of protein concentration

Protein concentrations of purified Bt2, Bt3, Bt73 and Bt15 toxins were calculated from the OD at 280 nm (measured with a Uvikon 810 P, Kontron Instruments spectrofotometer). The protein content of solutions of other toxins and of brush border membrane vesicles (BBMV) as measured according to Bradford (1976).

Binding of Bt2, Bt3 and Bt73 toxins to BBMV of Manduca sexta and Heliothis virescens: an example of 3 competitively binding Lepidopteran ICPs.

Bt2, Bt3 and Bt73 toxins are toxic to both Manduca sexta and Heliothis virescens: LC50 values for Manduca sexta are respectively 17.70, 20.20 and 9.00 ng/cm2 ; for Heliothis virescens the LC50's are 7.16, 90.00 and 1.60 ng/cm2.

Labelled toxin, either Bt3 (0.8 nM) or Bt2 (1.05 nM) or Bt73 (1.05 nM), was incubated with BBMV in a volume of 0.1 ml. BBMV protein concentrations were 100 ug/ml for M. sexta and for Bt2-H. virescens, for Bt3-H.

virescens 150 and for Bt73-H. virescens 50 ug/ml. The labelled toxin was combined with varying amounts of an unlabeled toxin (competitor). After a 30 min. incubation, bound and free toxins were separated through filtration.

Figs. 1-3 show the percentages binding of respectively labelled Bt2, Bt3 and Bt73 toxins as a function of the concentration of competitor for Manduca sexta. Figs. 4-6 show these data for Heliothis virescens. The amount bound in the absence of competitor is always taken as 100 % binding. Figs. 1-6 show the binding of ^{125}I-labeled toxins to M. sexta (in Figs. 1, 2 and 3) and H. virescens (in Figs. 4, 5 and 6) brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Figs. 1 and 4: ^{125}I-Bt2-toxin (1.05nM); in Figs. 2 and 5: ^{125}I-Bt3-toxin (0.8nM); in Figs. 3 and 6: ^{125}I-Bt73-toxin (1.05nM)] in the presence of increasing concentrations of Bt2 toxin (*), Bt3 toxin (●) or Bt73 toxin (▲). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. On M. sexta vesicles, these amounts were 1820, 601 and 2383 cpm, and on H. virescens vesicles 1775, 472 and 6608 cpm for ^{125}I-Bt2-, Bt3- and Bt73-toxin, respectively. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample.

Figure 1: shows the binding of ^{125}I Bt2 toxin to M. sexta BBMV

Figure 2: shows the binding of ^{125}I Bt3 toxin to M. sexta BBMV

Figure 3: shows the binding of ^{125}I Bt73 toxin to M. sexta BBMV

Figure 4: shows the binding of ^{125}I Bt2 toxin to H. virescens BBMV

Figure 5: shows the binding of ^{125}I Bt3 toxin to H.virescens BBMV

Figure 6: shows the binding of ^{125}I Bt73 toxin to H.virescens BBMV

The conclusions from Figures 1-6 are that Bt2 and Bt3, Bt3 and Bt73, and Bt2 and Bt73 are competitively-binding ICP's both for Manduca sexta and for Heliothis virescens. Indeed Bt3 competes for the entire population of receptor sites of Bt2 in Manduca sexta (Fig.1): the % labelled Bt2 bound in the presence of 100 nM Bt3 is equal to the % Bt2 bound with 100 nM of Bt2 itself. The opposite is not true: in the presence of 100 nM Bt2 the % of labelled Bt3 is not reduced to the same level as with 100 nM of Bt3 (Fig.2).

A similar reasoning is followed to observe competitivity of other toxin combinations : Bt3 competes for the entire population of receptor sites of Bt73 (Fig. 3) in M. sexta; the opposite is not true (Fig. 2); Bt2 and Bt73 compete for the entire population of each other's binding sites in M. sexta (Figs. 1 and 3).

In Heliothis virescens : Bt2 competes for the entire population of receptor sites of Bt3 (Fig. 5); Bt73 competes for the entire population of receptor sites of Bt3 (Fig. 5); Bt73 competes for the entire population of receptor sites of Bt2 (Fig. 4); but the opposite statements are not true (Figs. 4, 5 and 6).

The same data can be used in mathematical analysis (e.g., Scatchard analysis according to Scatchard, 1949; analysis with the LIGAND computer program according to Munson and Rodbard, 1980) to calculate the dissociation constant (Kd) of the toxin-receptor complex and the concentration of binding sites (Rt); the results of these calculations using the LIGAND computer program were the following:

Bt2-M.sexta: Kd=0.4 nM Rt=3.4 pmol/mg vesicle protein

Bt3-M. sexta: Kd=1.5 nM Rt=9.8 pmol/mg vesicle protein

Bt73-M. sexta: Kd=0.6 nM Rt=4.0 pmol/mg vesicle protein

Bt2-H. virescens: Kd=0.6 nM Rt=9.7 pmol/mg vesicle protein

Bt3-H. virescens: Kd=1.2 nM Rt=3.7 pmol/mg vesicle protein

Bt73-H. virescens: Kd=0.8 nM Rt=19.5 pmol/mg vesicle protein

These data demonstrate the high affinity receptor binding of the toxins (Kds in the range of 10^{-10} to 10^{-9} M.

Binding of Bt2 and Bt14 toxins to BBMV of P. brassicae, Plutella xylostella and Phthorimaea operculella: an example two non-competitively binding Lepidopteran ICPs

Bt2 and Bt14 toxins are toxic to P. brassicae (p.b.), P. xylostella (p.x.) and P. operculella (p.o.) as seen from the table below.

	LC_{50} of Toxins	
	Bt2	Bt14
P.b.	1.3	2.0
P.x.	6.7	5.4
P.o.	4.20	0.8-4.0

LC_{50} values of solubilized purified Bt2 and Bt14 toxins for P.x. are expressed as ng protein spotted per cm^2 of artificial diet. LC_{50} values for P.b. are expressed as ug^2 toxin per ml solution into which leaf discs, fed to first instar Pb larvae, were dipped. For P.o., LC_{50} values are expressed in ug/ml into which potato chips were dipped prior to feeding.

Labelled Bt2 toxin (1.05 nM) or Bt14 toxin (1.4 nM) was incubated with BBMV from P. brassicae (100 ug

protein/ml) in a volume of 0.1 ml in combination with varying amounts of unlabelled Bt2 or Bt14. After a 30 min. incubation period at 22°C, the bound and free toxins were separated.

Figures 7 and 8 show the binding of ^{125}I-labeled toxins to P. brassicae brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Fig. 7: ^{125}I-Bt2-toxin (1.05nM); in Fig. 8: ^{125}I-Bt14-toxin (1.4nM)] in the presence of increasing concentrations of Bt2 toxin (o) or Bt14 toxin (•). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample. Figure 7 shows the binding of labelled Bt2 toxin to P. brassicae BBMV, and Figure 8 shows the binding of labelled Bt14 toxin to P. brassicae BBMV.

The competition data demonstrate the presence of high affinity binding sites both for Bt2 and Bt14, as well as the almost complete absence of competition of Bt14 for the Bt2 binding sites and of Bt14 for the Bt2 binding sites. This demonstrates that Bt2 and Bt14 are non-competitively binding toxins. Hence they are useful to prevent the development of Pieris brassicae resistance against B. thuringiensis ICP's expressed in Brassica sp.

Calculated Kd and Rt values were from these experiments were:

Bt2: Kd=2.8 nM, Rt=12.9 pmol/mg vesicle protein

Bt14: Kd=8.4 nM, Rt=21.4 pmol/mg vesicle protein.

Binding of Bt2 and Bt15 toxins to BBMV of M.sexta, M.brassicae, P. xylostella and P.interpunctella : an example of two non-competitively binding Lepidopteran ICPs

Bt2 and Bt15 toxins are both toxic to M.sexta (LC50's of 20 and 111 ng/cm2, respectively). They also show activity against M. brassicae, P. xylostella and P. interpunctella.

Labelled Bt2 (1.05 nM) or Bt15 (0.7 nM) was incubated with BBMV from M.sexta (100 ug protein/ ml) in a volume of 0.1 ml in combination with varying amounts of unlabelled Bt2 or Bt15. After a 30 min. incubation period at 22°C, the bound and free toxins were separated.

Figs. 9-10 show the binding of ^{125}I-labeled toxins to M. sexta brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Fig. 9: ^{125}I-Bt2-toxin (1.05nM); in Fig. 10: ^{125}I-Bt15-toxin (0.7nM)] in the presence of increasing concentrations of Bt2-toxin (o) or Bt15-toxin (●). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample. Figure 9 shows the data for binding of labelled Bt2, and Figure 10 shows the binding of labelled Bt15.

The competition data demonstrate the presence of high affinity binding sites for both Bt2 and Bt15, as well as the complete absence of competition of Bt15 for the Bt2 binding sites and of Bt2 for the Bt15 binding sites. This demonstrates that Bt2 and Bt15 are non-competitively binding toxins. Hence the combination of Bt2 and Bt15 is useful to prevent the development of resistance of M.sexta against B. thuringiensis ICP's expressed in tobacco or other crops in which Manduca sp. are a pest. Calculated Kd and Rt values are:
Bt2: Kd=0.4 nM, Rt=3.4 pmol/mg vesicle protein.

Bt15: Kd = 0.3 nM Kd2=2.9 nM, Rt1= 5.9 and Rt2=6.7 pmol/mg vesicle protein (2 distinct high affinity receptor sites are present).

Similar studies were performed for M. brassicae, S. littoralis and P. interpunctella. Although LD50, Kd and Rt values differed substantially, the essential observation that Bt2 and Bt15 are both toxic and are non-competitively binding toxins was confirmed in these three insect species. Thus, it is also a useful toxin combination to prevent resistance of M. brassicae to ICP's or to prevent resistance of Spodoptera species against ICP's expressed in any of the crop plants in which Spodoptera species are a pest.

Binding of Bt2 and Bt4 toxins to BBMV of M. sexta: an example of two non-competitively binding Lepidopteran ICPs

Both Bt2 and Bt4 toxins are toxic to Manduca sexta. LD50 values are 20 and 5.4. ng/cm2, respectively. No mutual competition of Bt2 for binding of labelled Bt4 and of Bt4 for binding of labelled Bt2 was observed, demonstrating that Bt2 and Bt4 are non-competitively binding toxins.

Binding of Bt15 and Bt18 toxins to BBMV of S. littoralis: an example of two non-competitively binding Lepidopteran ICPs

Both Bt15 and Bt18 toxins are toxic to S. littoralis. LD 50 values are 93 and 88 ng toxin/cm^2, respectively. Labelled Bt15 (0.7 nM) or Bt18 (0.9 nM) was incubated with 100 ug of vesicle protein from S. littoralis in combination with varying amounts of unlabelled Bt15 or Bt18 toxin. After a 45 - min. incubation period, bound and free toxins were separated. Binding data demonstrate high affinity binding for both Bt15 and Bt18 to S. littoralis BBMV. As seen from Figures 11 and 12, the entire population

of receptor sites of Bt15 was not saturable with Bt18, nor was the entire population of receptor sites of Bt18 saturable with Bt15.

Binding of Bt13 and Bt22 toxins to BBMV of L. decemlineata : an example of two non-competitively binding Coleopteran ICPs.

Both Bt13 and Bt22 toxins are toxic to L. decemlineata. LD 50 values are 0.8 and 1.1 ug toxin/ml respectively. Labelled Bt13 (1 nM) or Bt22 (0.7 nM) was incubated with 100 ug of vesicle protein/ml from S. littoralis in combination with varying amounts of unlabelled Bt13 or Bt22 toxin. After a 45 min. incubation period, bound and free toxins were separated. Binding data demonstrate high affinity binding for both Bt13 and Bt22 to S. littoralis BBMV. The entire population of receptor sites of Bt13 was not saturable with Bt22. Nor was the entire population of receptor sites of Bt22 saturable with Bt13.

Binding of Bt2 and Bt18 toxins to BBMV of M. sexta: an example of two non-competitively binding Lepidopteran ICPs.

Both Bt2 and Bt18 toxins are toxic to M. sexta, and LD 50 values are 20 to 73 ng toxin/cm^2 respectively. Labelled Bt2 (1.05nM) or Bt18 (0.7nM) was incubated with 100 ug/ml of vesicle protein from M. sexta in combination with varying amounts of unlabelled Bt2 or Bt18 toxin. After a 45 min. incubation period, bound and free toxins were separated. Binding data (Figs. 11-12) demonstrate high affinity binding for both Bt2 and Bt18 to M. sexta BBMV. The entire population of receptor sites of Bt2 was not saturable with Bt18. Nor was the entire population of receptor sites of Bt18 saturable with Bt2. Calculated Kd and Rt values are:

Bt2: Kd= 0.4 nM, Rt= 3.4 pmol/mg vesicle protein.

Bt18: Kd1= 0.04 nM, Rt1= 2.2 pmoles/mg vesicle protein and Kd2= 168nM Rt2= 194 pmoles/mg vesicle protein (2 distinct receptor sites for Bt18 are present).

A list of non-competitively binding anti-Lepidopteran ICP combinations and anti-Coleopteran ICP combinations is given below, together with their common target insect species in which non-competitivity has been demonstrated:

Bt2-Bt15 (Manduca sexta, Plutella xylostella, Pieris brassicae, Mamestra brassicae, Plodia interpunctella)

Bt2-Bt18 (Manduca sexta, Spodoptera littoralis)

Bt2-Bt14 (Pieris brassicae, Plutella xylostella, Phthorimaea operculella)

Bt2-Bt4 (Manduca sexta)

Bt15-Bt18 (Manduca sexta, Spodoptera littoralis)

Bt14-Bt15 (Pieris brassicae)

Bt15-Bt4 (Manduca sexta, Spodoptera exigua)

Bt18-Bt4 (Manduca sexta, Spodoptera littoralis)

Bt18-Bt14 (Pieris brassicae)

Bt18-Bt4 (Manduca sexta)

Bt13-Bt21 (Leptinotarsa decemlineata)

Bt13-Bt22 (Leptinotarsa decemlineata)

Bt21-Bt22 (Leptinotarsa decemlineata)

Of course, this list of specific non-competitively binding ICP combinations for specific target insect pests is not exhaustive, and it is believed that other such ICP combinations, including combinations for yet-to-be discovered ICPs, will be found using a similar approach for any target insect species. Likewise, the foregoing list of target insect pests also is not exhaustive, and it is believed that other target insects pests (as well as the plants that are to be transformed to prevent their attack by such pests), against which the specific combinations of ICPs can be

used (e.g., the combination of the Bt2 and Bt14 ICPs in Brassica to prevent resistance of Pieris brassicae against the ICPs expressed in the plant), will be found using a similar approach.

EXAMPLE 7: Selection for resistance of Manduca sexta (tobacco hornworm)

A selection experiment involves exposing a large number of larvae to a concentration of a toxin in a diet killing (e.g., 50-90 %) of the larvae. The surviving larvae are again exposed to toxin concentrations killing a similar proportion of the larvae, and this process is continued for several generations. The sensitivity of the larvae to the toxin is investigated after each four generations of selection.

Selections for 20 generations of M. sexta were performed with Bt2 toxin alone, with Bt18 toxin alone and with a 1/4 (by weight) Bt2/Bt18 mixture. LC50 values of the reference strain for Bt2, Bt18 and the 1/4 Bt2/Bt18 mixture respectively were the following : 20 ng/cm2, 73 ng/cm2 and 62 ng/cm2 of diet.

Selection was initiated at concentrations killing around 75 % of the larvae. After 4 generations of selection, survival increased in both the Bt2 and the Bt18 selection to around 70 %, no such increase was observed in the selection with the combination of Bt2 and Bt18. Dosages were again increased to calculated LC75 values. This was repeated every 4 generations. The selection process was thus continued to the 20th generation. Final results were the following (LC50 of the 20th generation):

- Bt2 selection: LC50 was 6400 ug/g (320 times decreased sensitivity)
- Bt18 selection: LC50 was 15100 ug/g (207 times decreased sensitivity)

- Bt2/Bt18 selection: LC50 was 181 ug/g (3 times decreased sensitivity).

Thus the decrease in sensitivity was about 100 times slower in the combined selection experiment.

Receptor binding in the three selected M. sexta strains was investigated with Bt2 and Bt18 and compared to those of the reference M. sexta strain (non-selected strain). Binding characteristics of the reference strain for the Bt2 and BT18 toxins were:

Bt2: Kd = 0.4 nM, Rt=3.4 pmol/mg vesicle protein

Bt18: Kd1=0.04 nM, Rt1=2.2 pmoles/mg vesicle protein and Kd2=168nM, Rt2=194 pmoles/mg vesicle protein (2 distinct receptor sites for Bt18 are present).

Figures 11 and 12 show the binding of ^{125}I-labeled toxins to M. sexta brush border membrane vesicle. Vesicles were incubated with labeled toxin [in Fig. 11: ^{125}I-Bt2-toxin (1.05nM); in Fig. 12: ^{125}I-Bt18-toxin (0.7nM)] in the presence of increasing concentrations of Bt2-toxin (o) or Bt18-toxin (•). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample.

The Bt2 selected strain showed no detectable high affinity binding of Bt2 whereas its Bt18 binding characteristics remained close to the reference strain. (Bt18: Kd1=0.03 nM, Rt1=2.8 pmoles/mg vesicle protein and Kd2=199nM, Rt2=109 pmoles/mg vesicle protein; 2 distinct receptor sites for Bt18 are still present).

The Bt18 selected strain lost the high affinity receptor site for Bt18. The lower affinity site for Bt18 was still present in lower concentration than in the reference strain (Kd=189 nM, Rt=43 nM). Bt2 binding site concentration increased markedly compared to the

reference strain (Kd=0.4 nM, Rt=20.8 pmoles/mg vesicle protein). This strain had a Bt2 sensitivity of LC_{50}=4 ng/cm^2. Thus, its sensitivity for Bt2 had increased as compared to the reference strain (LC_{50}=20 ng/cm^2).

The Bt2/Bt18 selected strain showed a slight but statistically non-significant decrease in Bt18 binding site concentration. (Bt2 : Kd = 0.4 nM, Rt=3.4 pmol/mg vesicle protein, Bt18 : Kd1=0.04 nM, Rt1=1.0 pmoles/mg vesicle protein and Kd2=168nM, Rt2=194 pmoles/mg vesicle protein; 2 distinct receptor sites for Bt18 are present). These data demonstrate that, in the two selection lines where resistance occurred, the mechanism was situated at the receptor level. Changes in receptor site are shown to be the most likely mechanism of resistance to B. thuringiensis ICPs.

EXAMPLE 8: Mechanism of resistance of the diamondback moth to the microbial insecticide Bacillus thuringiensis.

The mechanism of development of insect resistance to ICPs has been investigated in a P. xylostella strain ("PxR"). This insect strain has developed a high level of resistance in the field against Dipel. Crystals of Dipel preparations contain a mixture of ICPs such as Bt3, Bt2 and Bt73 ICPs; in Example 6, it has been shown that these toxins are competitively binding ICPs.

Resistance to Dipel was confirmed by the toxicity data for the sensitive strain ("PxS") and for the Dipel-resistant strain ("PxR"). High levels of resistance are also observed for the Bt2 protoxin and toxin as shown in the following table :

	LC_{50} of Strains	
	PxS	PxR
Bt2	6.7	> 1350
Bt15	132.6	120.4

LC_{50} data are expressed as ng protein spotted per cm^2 of artificial diet.

However, insect toxicity data show that there is no resistance to the Bt15 protoxin and Bt15 toxin; this ICP is not present in Dipel crystals. To investigate whether a change in toxin-membrane binding was responsible for resistance, receptor binding studies were performed with ^{125}I-labeled Bt2 toxin and Bt15 toxin, with BBMV derived from larvae midguts of the PxR and PxS strains. The results are summarized in Table 1, below.

Table 1. Binding characteristics of Bt2 and Bt15 toxins to brush border membrane vesicles from sensitive and resistant P. xylostella.

ICP	strain	Kd (nM)	Rt (pmol/ mg protein)
Bt2 toxin	PxS	8.1	1.6
	PxR	no binding detectable	
Bt15 toxin	PxS	1.9	4.2
	PxR	3.7	5.8

Table 1 shows that there was high-affinity saturable binding of the Bt2 toxin to midgut membranes of the PxS strain, but the PxR strain showed no detectable level of Bt2 toxin binding. With the Bt15 toxin, there was significant binding to BBMW of both the PxR and PxS strains, and values are not significantly different for the two strains.

These data show that resistance in P. xylostella is due to an alteration in toxin-membrane binding. Resistance to the Bt2 toxin and the sensitivity toward the Bt15 toxin of the PxR strain is reflected by the binding characteristics shown in Table 1.

Hence, when different non-competitively binding ICPs (i.e., Bt2 and Bt15) are available with activity against the same insect species (e.g., P. xylostella),

resistance to one ICP(Bt2) does not imply resistance against other ICPs (such as Bt15). Thus, ICPs with different binding properties can be used in combination to delay development of insect resistance to ICPs.

EXAMPLE 9: Separate transfer of two ICP genes within individual transcriptional units to the genome of plant cells

Two procedures are envisaged for obtaining the combined expression of two ICP genes, such as the bt2 and bt15 genes in transgenic plants, such as tomato plants. These procedures are based on the transfer of two chimeric ICP genes, not linked within the same DNA fragment, to the genome of a plant of interest.

A first procedure is based on sequential transformation steps in which a plant, already transformed with a first chimeric ICP gene, is retransformed in order to introduce a second ICP gene. The sequential transformation makes use of two different selectable marker genes, such as the resistance genes for kanamycin ("km") and phosphinotricin acetyl transferase ("PPT"), which confers resistance to phoshinotricin. The use of both these selectable markers has been described in De Block et al. (1987).

The second procedure is based on the cotransformation of two chimeric ICP genes on different plasmids in a single step. The integration of both ICP genes can be selected by making use of the two selectable markers conferring resistance to Km and PPT, linked with the respective ICP genes.

For either procedure, a Ti-plasmid vector is used for Agrobacterium-mediated transformation of each chimeric ICP gene into plant cells.

Plasmid pGSH163, described in EP 0193259, contains the following chimeric genes between the T-DNA border

repeats: a gene fragment encoding the toxin part of the bt2 gene under the control of the TR2' promoter and the neo gene under control of the TR1' promoter. The 3' ends of the T-DNA gene 7 and octopine synthase respectively provide information for the 3' end formation of transcripts.

A chimeric bt15 gene containing a gene fragment encoding the toxin of the Bt15 ICP under the control of the TR2' promoter, was constructed in the following way (Figure 15). pOH50 consists of pUC18 with the whole bt15 gene under the control of the lac promoter. A HindIII-BglII fragment was cloned in pMa5-8 yielding pJB3. By site-directed mutagenesis, a NcoI site was created at the initiation codon to yield pVE29. A fragment containing the truncated gene fragment of the bt15 gene, with a translational stop codon, was obtained by isolation of BclI-ClaI from pOH50 and cloning in pLK91, yielding pHW38. The whole toxin gene fragment was reconstructed under the control of the tac promoter, yielding pVE35, by ligation of a ClaI-PstI fragment from pHW38, a NcoI-ClaI fragment from pVE29 and a NcoI-PstI fragment from pOH48. A truncated bt15 gene fragment with a NcoI site at the initiation codon was obtained from pVE35 as a 1980 NcoI-BamHI fragment and cloned in pGSJ141, digested with ClaI and BamHI. pGSJ141 has been described in EPA 88402115.5. Ligation of the filled ClaI site to the filled NcoI site yielded a chimeric TR2' - truncated bt15 - 3'g7 construct (pTVE47). As a selectable marker in this plasmid, the bar gene encoding phosphinothricin acetyl transferase and conferring resistance to PPT was used. A chimeric bar gene containing the bar gene under the control of the 35S promoter and followed by the 3' end of the octopine synthase was introduced in pTVE47. From pDE110, a 35S-bar-3'ocs fragment was obtained as a

StuI-HindIII fragment and was cloned in pTVE47 digested with PstI and HindIII. This yielded the plasmid pTHW88 (Figure 15) which contains the truncated bt15 gene under the control of the TR2' promoter and the bar gene under the control of the 35S promoter between the T-DNA border repeats. Plasmid pGSH163 is cointegration type Ti-plasmid vector, whereas pTHW88 is a binary type Ti-plasmid vector as described in EPA 0193259.

Both plasmids were mobilized in the A. tumefaciens strain C58C1Rif (pGV2260) according to Deblaere et al. (1988). In the sequential transformation procedure, tomato was transformed according to De Block et al. (1987) with the A. tumefaciens strain C58C1Rif carrying pGS1163 resulting from the cointegration of pGSH163 and pGV2260. Individual transformants were selected for kanamycin resistance, and regenerated plants were characterized for expression of the truncated bt2 gene according to Vaeck et al. (1987). One representative transformant was subsequently retransformed with the A. tumefaciens strain C58C1Rif (pGV2260 and pTHW88), and transformants were selected for PPT resistance. Using this cotransformation procedure, the respective Agrobacteria strains, carrying the cointegrate vector pGS1163 and the binary vector pTHW88, were used for transformation of tomato. Individual plants were selected for resistance to Km and PPT.

Schematically shown in Fig. 15 are:

a) construction of pVE29: bt15 N-terminal gene fragment with NcoI site introduced at ATG initiation codon.

b) construction of pVE35: bt15 C-terminal truncated gene fragment under control of the tac promoter.

c) construction of pTHW88: binary T-DNA vector with a chimeric bt15 gene and a chimeric bar gene within the T-DNA border repeats.

In both cases, co-expression of the two ICP genes in the individual transformants was evaluated by insect toxicity tests as described in EP 0193259 and by biochemical means. Specific RNA probes allowed the quantitive analysis of the transcript levels; monoclonal antibodies cross-reacting with the respective gene products allowed the quantitative analysis of the respective gene products in ELISA tests (EP 0193259); and specific DNA probes allowed the characterization of the genomic integrations of the bt2 and bt15 genes in the transformants. It was found that the transformed tomato plants simultaneously expressed both the bt2 gene (8.1 ng/mg) and the bt15 gene (7.6 ng/mg) as measured by ELISA, which would prevent or delay development of resistance of M. sexta to the insecticidal effects of the Bt2 and Bt15 toxins, being expressed.

These procedures also could be applied when one or both ICP genes are part of a hybrid gene. For example, the same strategy as described above could be followed with the plasmid vectors pGSH152, containing a chimeric truncated bt2-neo hybrid gene under control of the TR2' promoter, and pTHW88 in suitable Agrobacterium strains.

EXAMPLE 10: Separate transfer of two ICP genes to the nuclear genome of separate plants in independent transformation events and subsequent combination in a single plant through crossing.

Tobacco plants have been transformed with either the bt18 gene or the bt15 gene by applying the same cloning strategies as described in EP 0358557 and EP

193259, respectively. For both genes, the plants were transformed with plant expression vectors containing either the truncated bt18 or bt15 gene, which just encode the Bt18 or Bt15 toxin, respectively.

The mortality rate of Spodoptera littoralis larvae feeding on the transformed plants is significantly higher than the mortality rate of larvae fed on untransformed plants.

The bt18-transformed plant, which is homozygous for the bt18 gene, is then crossed with the bt15 - transformed plant, which is homozygous for the bt15 gene. After selfing, a plant homozygous for both genes is obtained.

The resulting tobacco plants, expressing both the bt18 and bt15 genes, delay significantly development of resistance by S. littoralis to either the Bt18 or Bt15 toxin expressed by the plants.

EXAMPLE 11: Transfer of two chimeric ICP genes linked within the same DNA to the genome of plant cells

The strategy used is based on the organization of two independent chimeric ICP genes between the T-DNA border repeats of a single vector. Binding studies indicated that the Bt2 and Bt14 toxins are two non-competitively binding ICPs with insecticidal activity towards Pieris brassicae. For expression in plants, both the bt2 and bt14 genes can be co-expressed to prevent insect resistance development. For the design of a plasmid vector with each ICP gene under the control of a separate promoter, two possibilities can be envisaged: 1) three chimeric constructs carrying the truncated bt2 and bt14 genes and a selectable marker, respectively; or 2) a hybrid of a truncated gene fragment (bt2 or bt14) and the neo gene can be used in combination with a truncated bt14 or bt2 gene.

This Example describes the construction of the vector pTHW94 for plant transformations carrying the following chimeric ICP genes between the T-DNA border repeats: a truncated bt2 gene fragment under the control of the TR2' promoter and a hybrid truncated bt14-neo gene under the control of the TR1' promoter. The 3' end of the T-DNA gene 7 and octopine synthase, respectively, provide information for proper 3' end formation. pTHW94 has been deposited at the DSM under accession no. 5514 on August 28, 1989.

Schematically shown in Fig. 16 are the:

a) construction of pHW44: bt14 N-terminal gene fragment with NcoI site introduced at ATG initiation codon.

b) construction of pHW67: reconstruction of the bt14 gene under the control of the tac promoter.

c) construction of pHW71: construction of a hybrid truncated bt14-neo gene under the control of the tac promoter.

d) construction of pTHW94: binary T-DNA vector with a chimeric bt14 gene and a chimeric bt2 gene within the T-DNA border repeats.

The pTHW94 vector is mobilized into the Agrobacterium strain C58C1Rif (pMP90) which is used to transform Brassica napus according to the procedure described by De Block et al. (1989). Transformants are selected on Km, and regenerated plants are found to express both ICP gene products in insect toxicity tests and biochemical tests.

EXAMPLE 12: Expression of two ICP genes in a hybrid construct

In order to obtain a combined and simultaneous expression of two ICP genes, truncated gene fragments encoding the toxic parts of two different ICPs can be fused in a proper reading frame and placed, as a hybrid gene, under the control of the same promoter in a chimaeric gene construct. Toxic cores from certain ICPs can be liberated from their protoxins by protease activation at the N- and/or C- terminal end. Thus, hybrid genes can be designed with one or more regions encoding protease cleavage site(s) at the fusion point(s) of two or more ICP genes.

The simultaneous co-expression of the bt2 and bt14 genes is obtained by constructing a hybrid gene composed of a truncated bt14 gene fragment fused to a truncated bt2 gene fragment. Schematically shown in Figure 17 is the construction of such a hybrid bt2-bt14 gene with a C-terminal bt2 gene fragment (bt860) encoding the toxic core of the Bt2 protoxin in frame with a C-terminal truncated bt14 gene fragment encoding the toxic core of the Bt14 protoxin. The BclI site in the bt2 gene, localized downstream of the trypsin cleavage site, is fused in frame with the NcoI site introduced at the N-terminal end of the truncated bt14 gene fragment. To this end, the plasmids pLBKm860 (EP 0193259) and pHW67 are used. pLBKm860 contains a hybrid bt2-neo gene under control of the lambda P_L promoter. The bt2 gene moiety in the hybrid gene is a C-terminal truncated bt2 gene fragment, indicated as bt860 (in Fig. 17) (see also Vaeck et al, 1987). The construction of pHW67 is described in Fig. 16. pHW67 contains a C-terminal truncated bt14 gene fragment (bt14tox) with a NcoI site at the ATG initiation codon, a

translation stop codon located at the BclI site of the intact bt14 gene and a BamHI site downstream of the whole gene fragment. To fuse both gene fragments in the proper reading frame, the BclI and NcoI ends of the respective plasmids are treated with Klenow DNA polymerase and S1 nuclease as indicated in Figure 16. The resulting plasmid pJB100 contains the hybrid bt860-bt14tox gene under control of the lambda P_L promoter and directs the expression in E. coli of a fusion protein with the expected mobility on SDS-PAGE.

Crude extracts of the E. coli strain show the toxicity of the fusion protein, expressed by the strain, against P. brassicae. It has also been confirmed by N-terminal amino acid sequence analyses of the fusion protein produced by the E. coli strain that the N-terminal amino acids from the Bt14 protoxin are processed upon activation. The bt2-bt14 hybrid gene product has thus two potential protease cleavage sites.

Subsequently, this hybrid gene is inserted into a vector for plant transformations and placed under control of a suitable promoter and transferred to the genome of brassica (EP 0193259) where both the bt2 and bt14 genes are expressed in insect toxicity tests.

Table 2

Gene	Bt strain	Host range	amino acids encoded	predicted MW(kDa) of encoded aminoacids	Disclosure of nucleotide sequence
bt3	HD-1 kurstaki	L	1176	133.2	Schnepf et al.,1985
bt2	berliner 1715	L	1155	131	Höfte et al.,1986
bt73	HD-73	L	1178	133.3	Adang et al, 1985
bt14	entomocidus HD-110	L	1207	138	Brizzard and Whiteley, 1988
bt15	entomocidus HD-110	L	1189	134.8	Fig. 14
bt4	HD-68 aizawai	L	1165	132.5	Fig. 13
bt18	darmstadiensis HD-146	L	1171	133	EP appln. 88402241.9
bt13	BtS1,DSM4288 22/10/87	C	644	73.1	EP appln. 88402115.5
bt21	BtPGSI208, DSM 5131, 19/1/89	C	651	74.2	EP appln. 89400428.2
bt22	BtPGSI245, DSM 5132, 19/1/89	C	1138	129	EP appln. 8940028.2
P2	HD-263	L/D	633	70.9	Donovan et al, 1988
Cry B2	HD-1	L	633	70.8	Widner and Whiteley, 1989

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CLAIMS

1. A cell of a plant, characterized by: at least two B. thuringiensis ICP genes stably inserted into the genome of said plant; each of said genes encoding a different non-competitively binding ICP for an insect species; whereby at least two different ICPs can be produced by said cell which do not bind competitively to the brush border membrane of the columnar midgut epithelial cell of said insect species.

2. The cell of claim 1 wherein at least one marker gene, encoding a protein or polypeptide which renders said cell easily distinguishable from cells which do not contain said protein or polypeptide, is in the same genetic locus as at least one of said ICP genes.

3. The cell of claim 1 or 2, wherein each of said ICP genes is under the control of a separate promoter capable of directing gene expression in said cell and is provided with a separate signal for 3' end formation and within a same transcriptional unit.

4. The cell of claim 2 or 3, in which said marker DNA is under the control of a separate promoter capable of directing gene expression in said plant cell and is provided with a signal for 3' end formation within a same transcriptional unit.

5. The cell of claim 1 or 2, wherein said ICP genes are within a same transcriptional unit and under the control of a single promoter.

6. The cell of claim 5, wherein said marker gene is fused with at least one of said ICP genes and is within said same transcriptional unit and under the control of said promoter.

7. The cell of claim 5 or 6, wherein a DNA fragment, encoding a protease-sensitive or -cleavable amino acid sequence, is in said same transcriptional unit as said ICP genes and intercalated in frame between said ICP genes.

8. The cell of claim 5 or 6, wherein said ICP genes are combined in a dicistronic unit comprising an intergenic DNA sequence which allows reinitiation of translation and is in said same transcriptional unit as said ICP genes and intercalated between said ICP genes.

9. The cell of anyone of claims 1 to 8, wherein said ICP genes are genes encoding insecticidal proteins having activity against Lepidoptera species and are particularly the following genes: bt2 and/or bt73 and/or bt4 and/or bt14 and/or bt15 and/or bt18.

10. The cell of any of claims 1 to 8, wherein said ICP genes are genes encoding insecticidal proteins having activity against a Coleoptera species and are particularly the following genes: bt13 and/or bt21 and/or bt22.

11. The cell of any of claims 2 to 10 wherein said marker DNA is: an herbicide resistance gene, particularly a sfr or sfrv gene; a gene encoding a modified target enzyme for a herbicide having a lower affinity for the herbicide, particularly a modified 5-EPSP as a target for glyphosate or a modified glutamine synthetase as a target for a GS inhibitor; or an antibiotic resistance gene, particularly NPTII.

12. The cell of any of claims 3 to 6, wherein said promoter is: a constitutive promoter, particularly a 35S promoter or a 35S3 promoter; a PNOS promoter; a POCS promoter; a wound-inducible

promoter, particularly a TR1' or TR2' promoter; a promoter which directs gene expression selectively in plant tissue having photosynthetic activity, particularly a SSU promoter; or a tissue-specific promoter, particularly a tuber-specific promoter, a stem-specific promoter or a seed-specific promoter.

13. A vector suitable for transforming a cell of a plant, particularly a plant capable of being infected with Agrobacterium, comprising said ICP genes of any of claims 1 to 12.

14. A process for producing a plant having improved insect resistance and having said ICP genes of anyone of claims 1 to 12 stably integrated into the nuclear genome of their cells, characterized by the non-biological steps of transforming a cell of said plant by introducing said ICP genes into the nuclear genome of said cell and regenerating said plant and reproduction material from said cell.

15. A plant cell culture, consisting of the plant cells of anyone of claims 1 to 12.

16. A plant, consisting of the plant cells of anyone of claims 1 to 12.

17. Brassica, tomato, potato, tobacco, cotton or lettuce consisting of the plant cells of anyone of claims 1 to 12, wherein said ICP genes comprise one of the following pairs of genes: bt2 and bt18 or bt73 and bt15 or bt2 and bt18 or bt2 and bt14 or bt2 and bt4 or bt15 and bt18 or bt14 and bt15 or bt4 and bt15 or bt13 and bt21 or bt21 and bt22 or bt13 and bt22.

18. The cell of anyone of claims 1-12, made by a process as described hereinabove.

19. A method for rendering a plant resistant to an insect species by transforming the plant with said ICP genes of anyone of claims 1-12.

ABSTRACT

Plants made resistant to insects by transforming their nuclear genome with two or more DNA sequences, each encoding a different non-competitively binding B. thuringiensis protoxin or insecticidal part thereof, preferably the toxin thereof.

FIG. 1

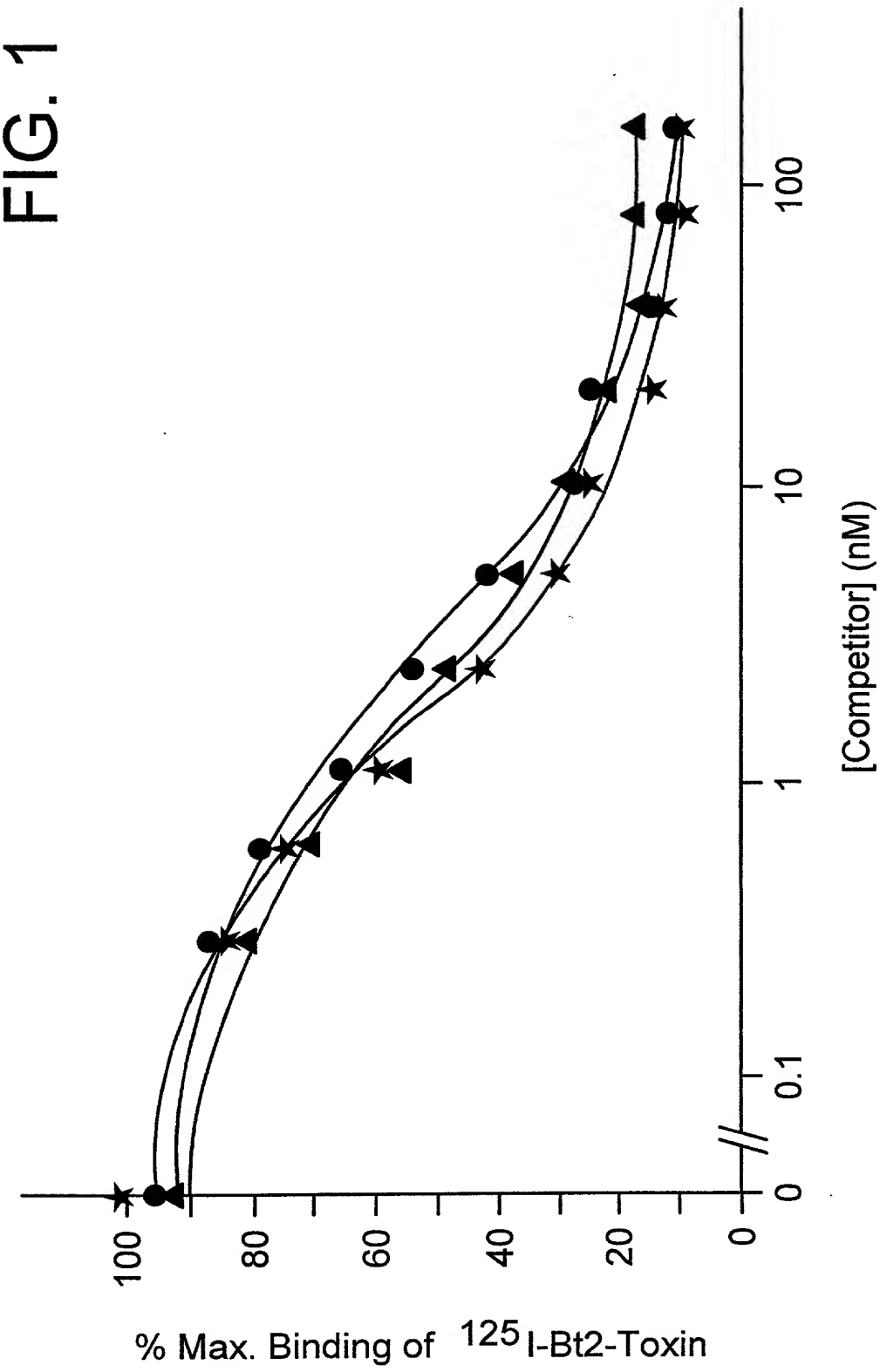

100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)
% Max. Binding of 125I-Bt2-Toxin

FIG. 2

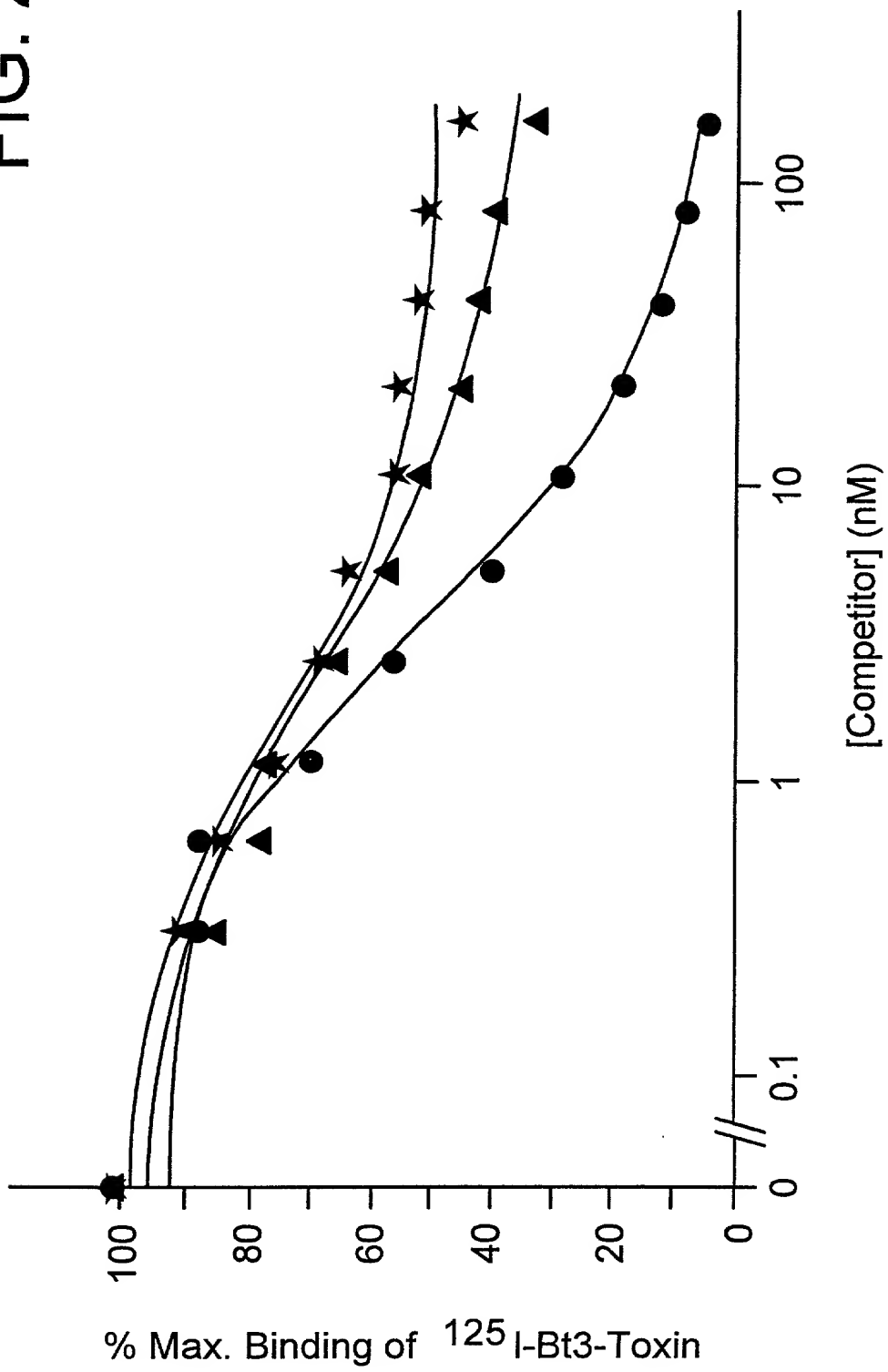

% Max. Binding of ^{125}I-Bt3-Toxin
100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

FIG. 3

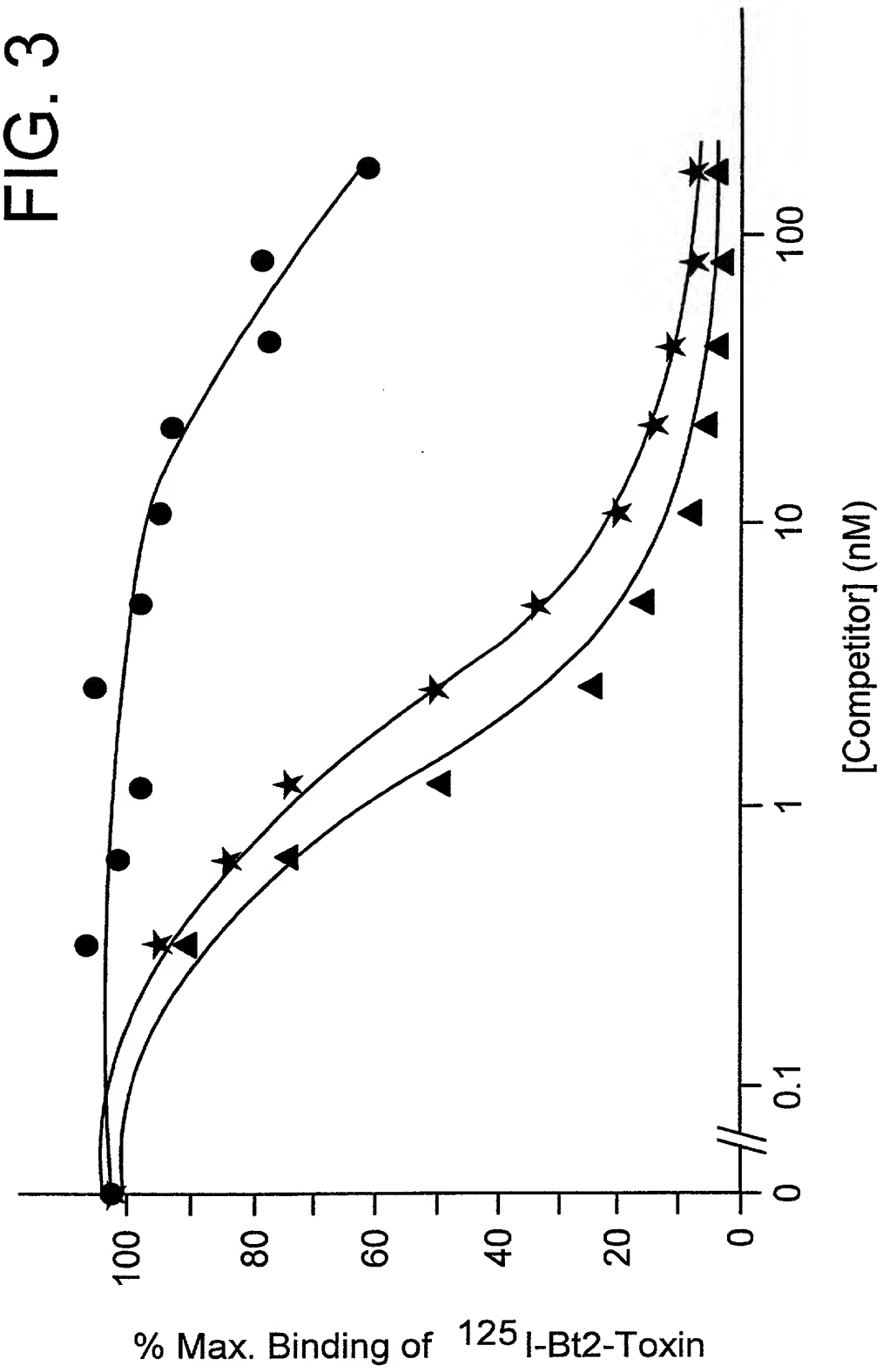

100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)
% Max. Binding of 125 I-Bt2-Toxin

FIG. 4

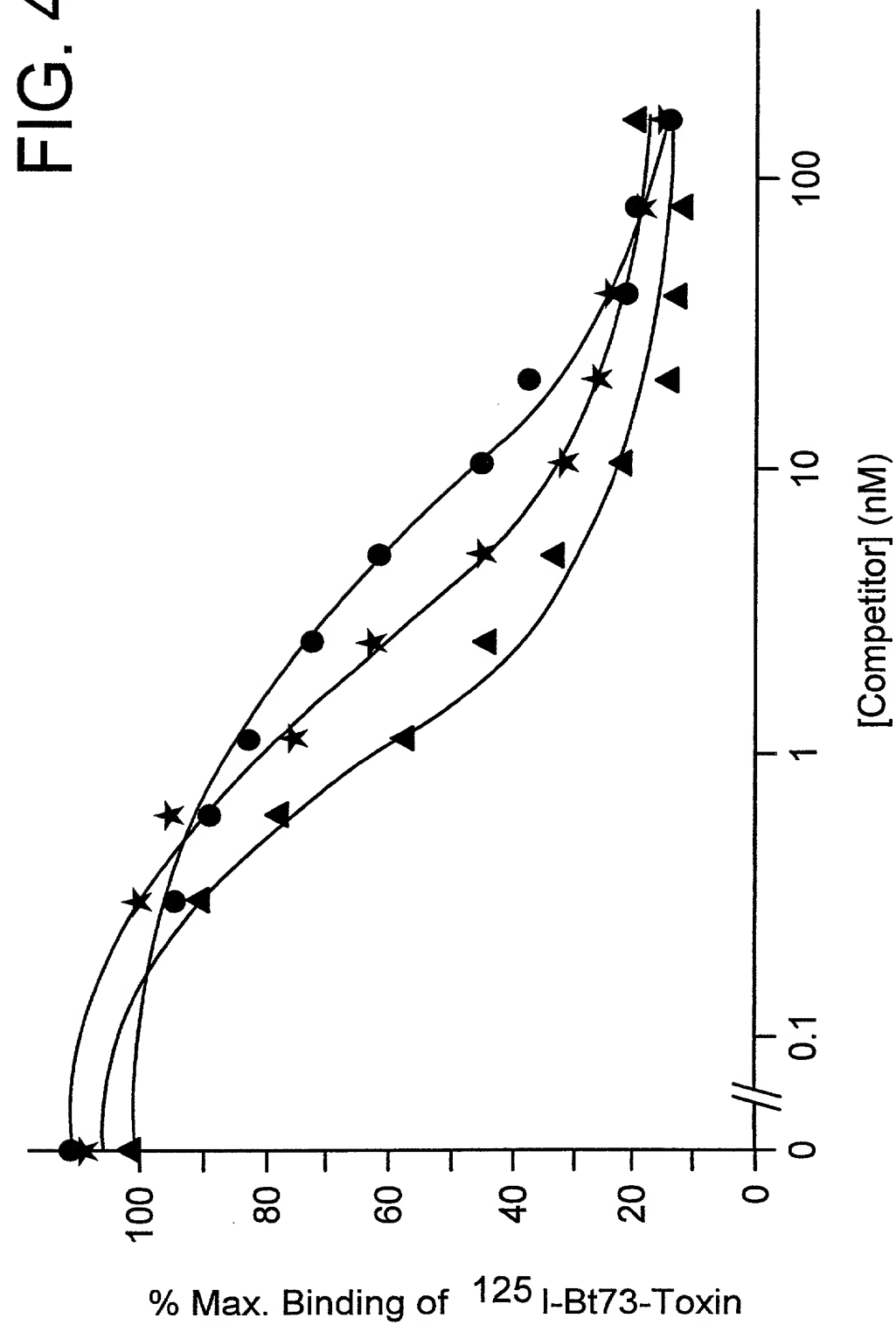
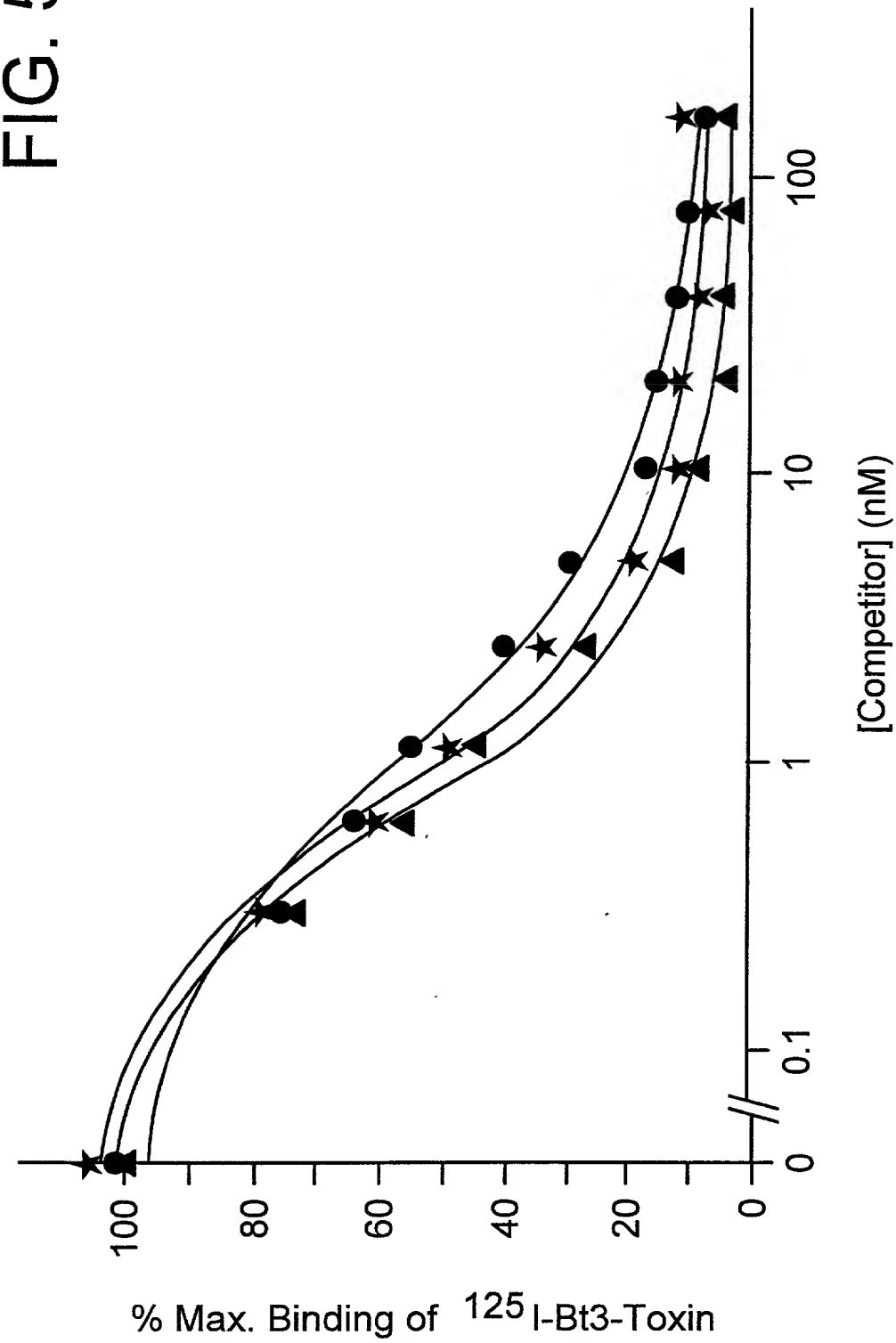

100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)
% Max. Binding of 125 I-Bt73-Toxin

FIG. 5


% Max. Binding of ^{125}I-Bt3-Toxin
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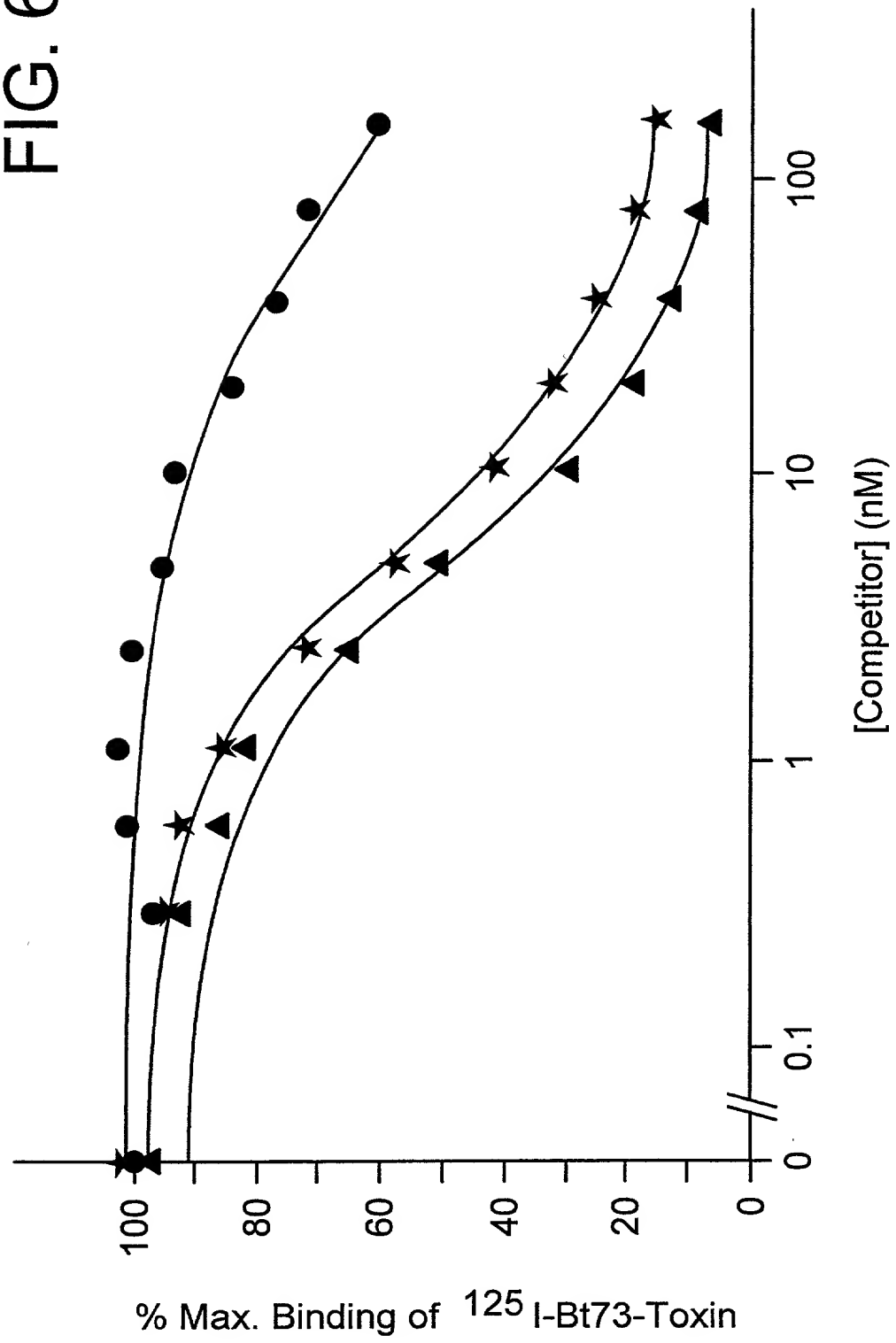
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

FIG. 6


% Max. Binding of ^{125}I-Bt73-Toxin
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80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

FIG. 7

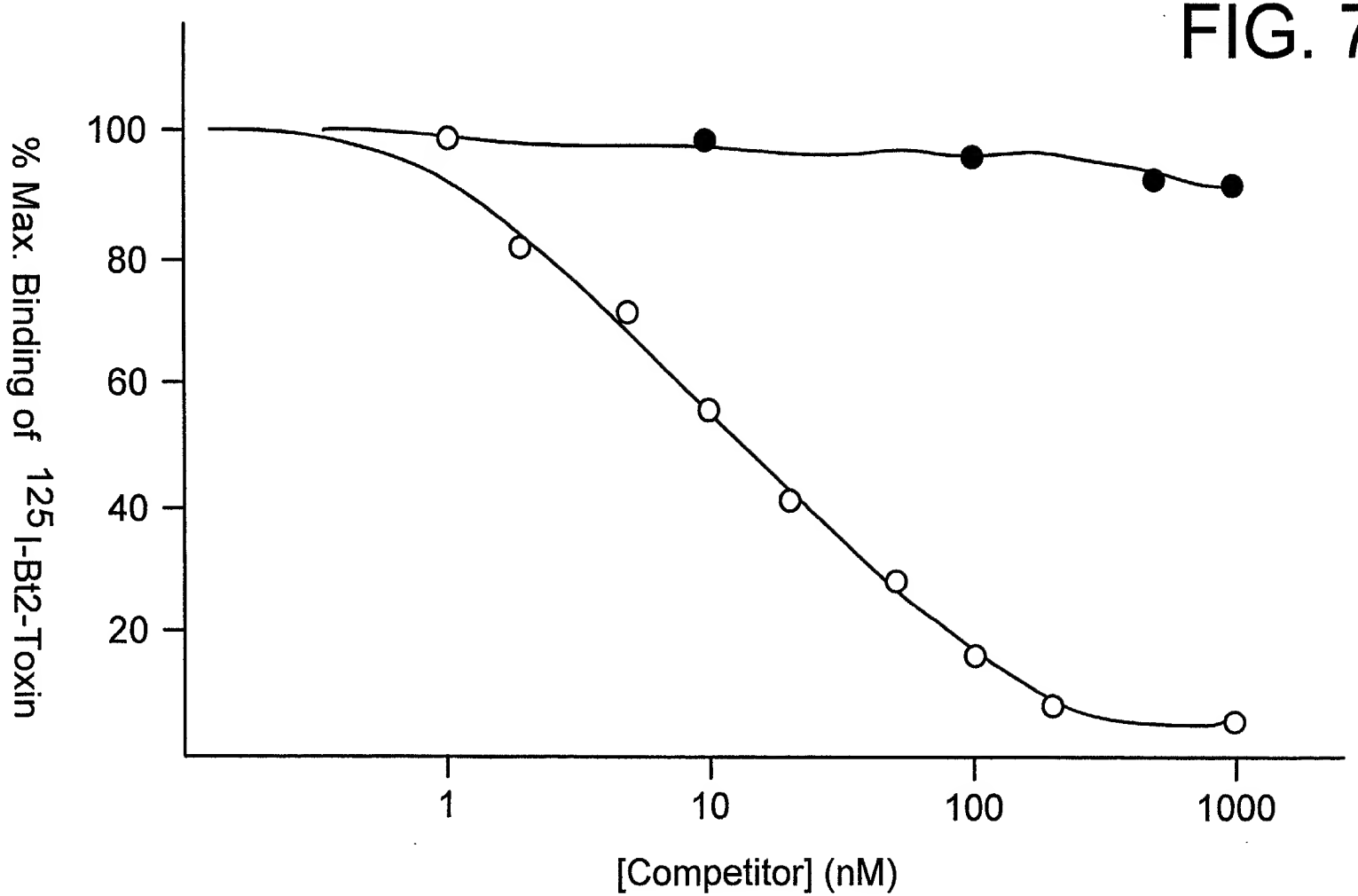


FIG. 8

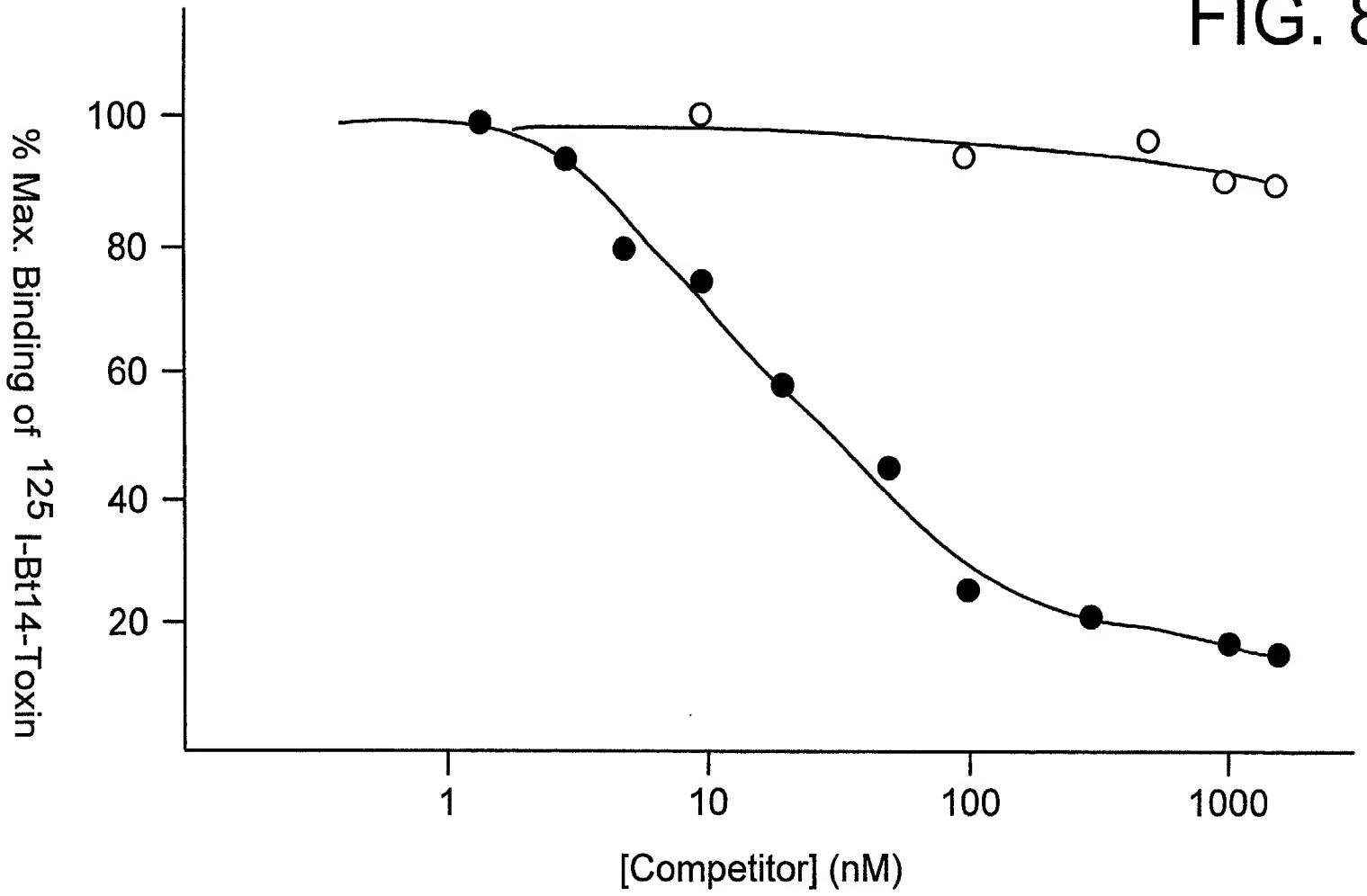


FIG. 9

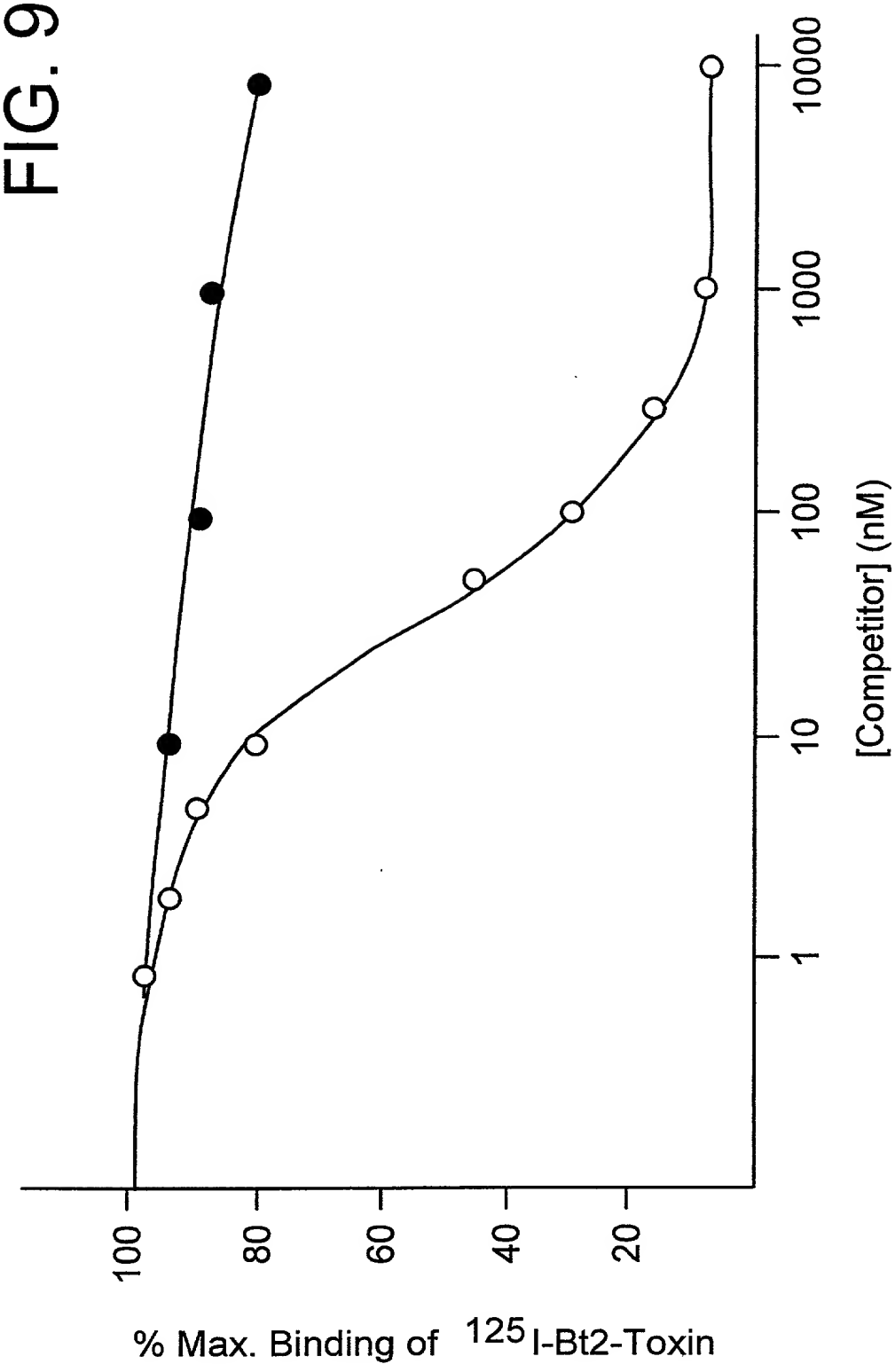

100
80
60
40
20
% Max. Binding of 125I-Bt2-Toxin
1
10
100
1000
10000
[Competitor] (nM)

FIG. 10

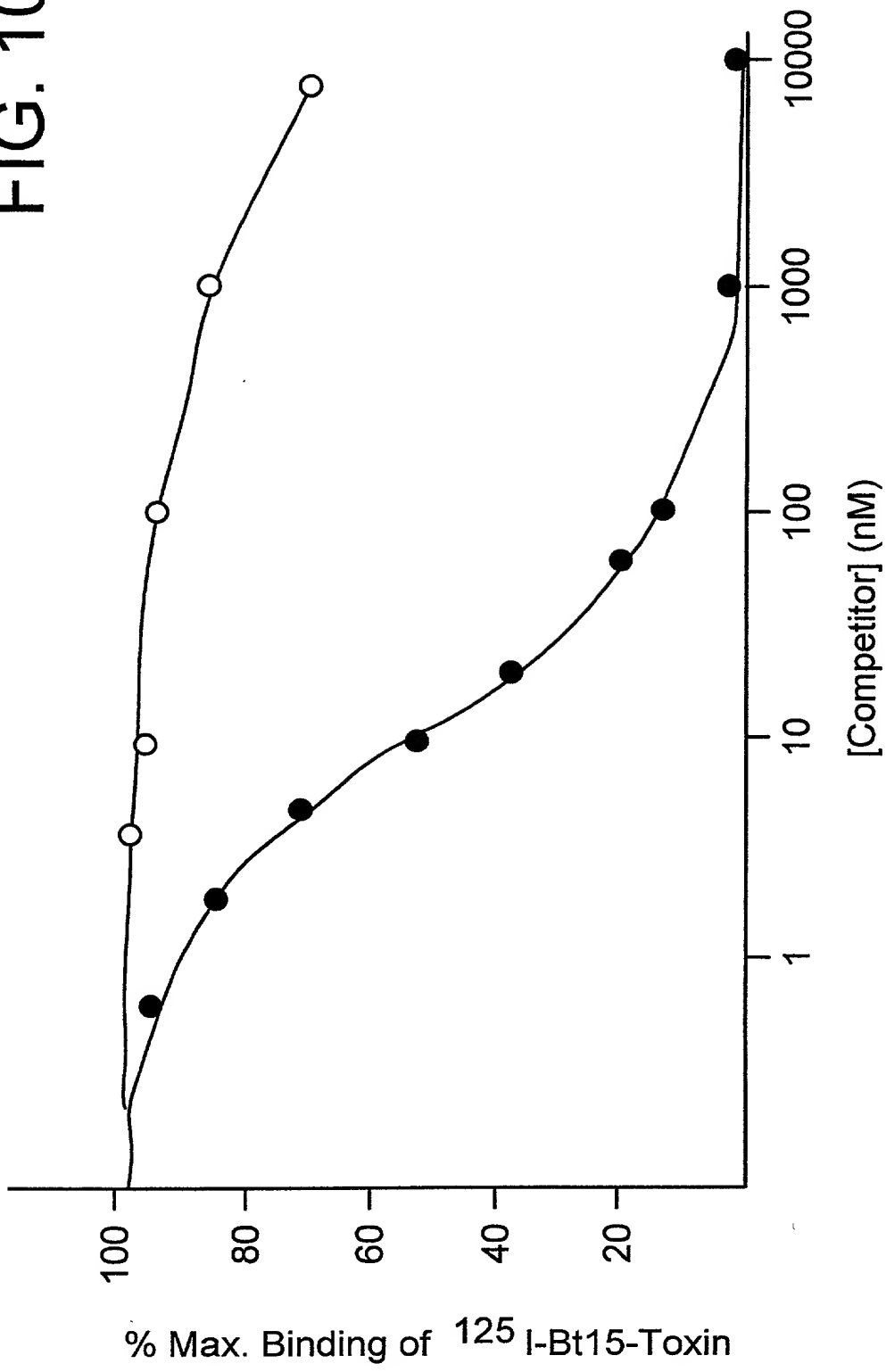

100
80
60
40
20
% Max. Binding of 125 I-Bt15-Toxin
1
10
100
1000
10000
[Competitor] (nM)

FIG. 11

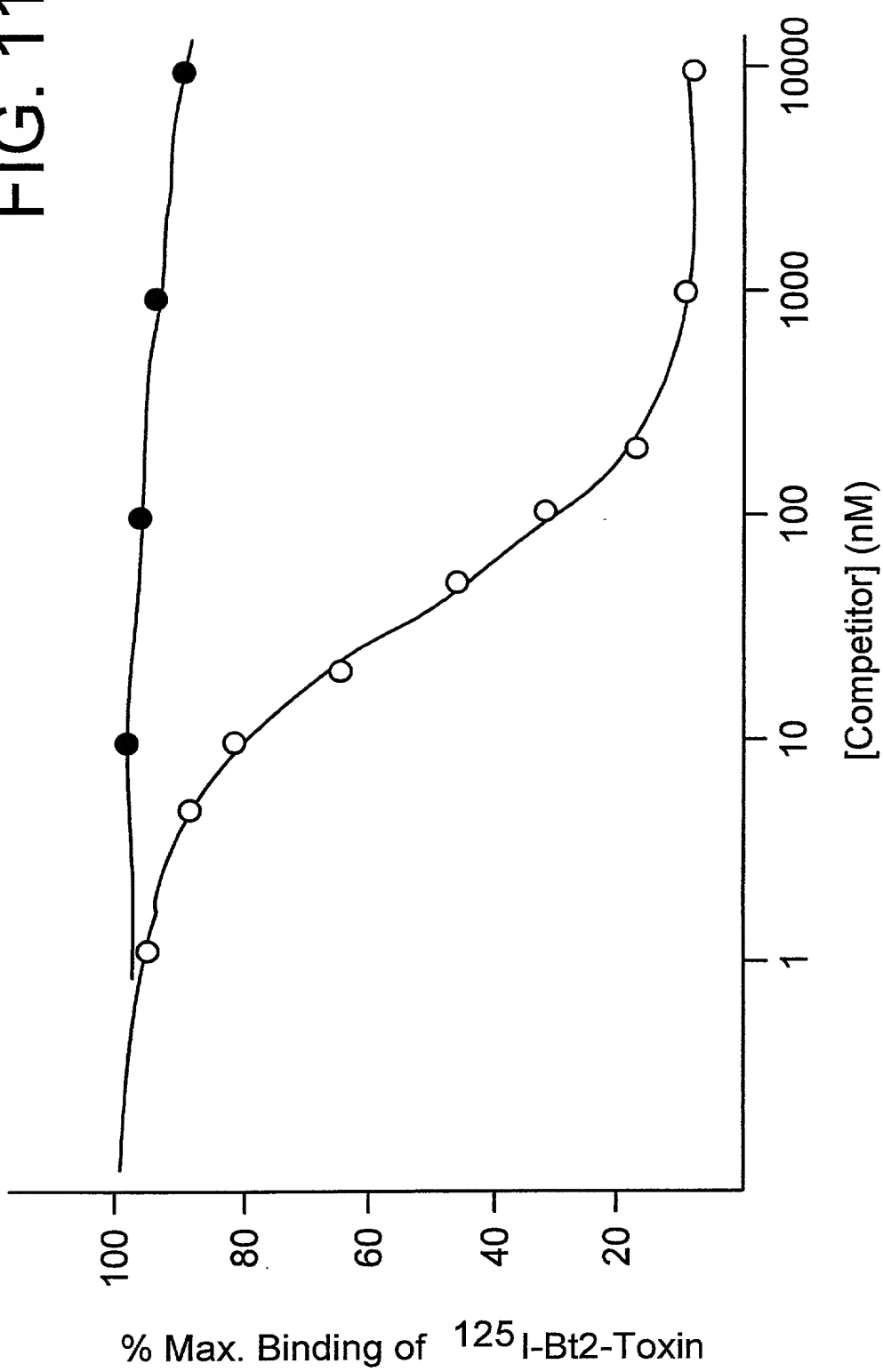

100
80
60
40
20
% Max. Binding of ^{125}I-Bt2-Toxin
1
10
100
1000
10000
[Competitor] (nM)

FIG. 12

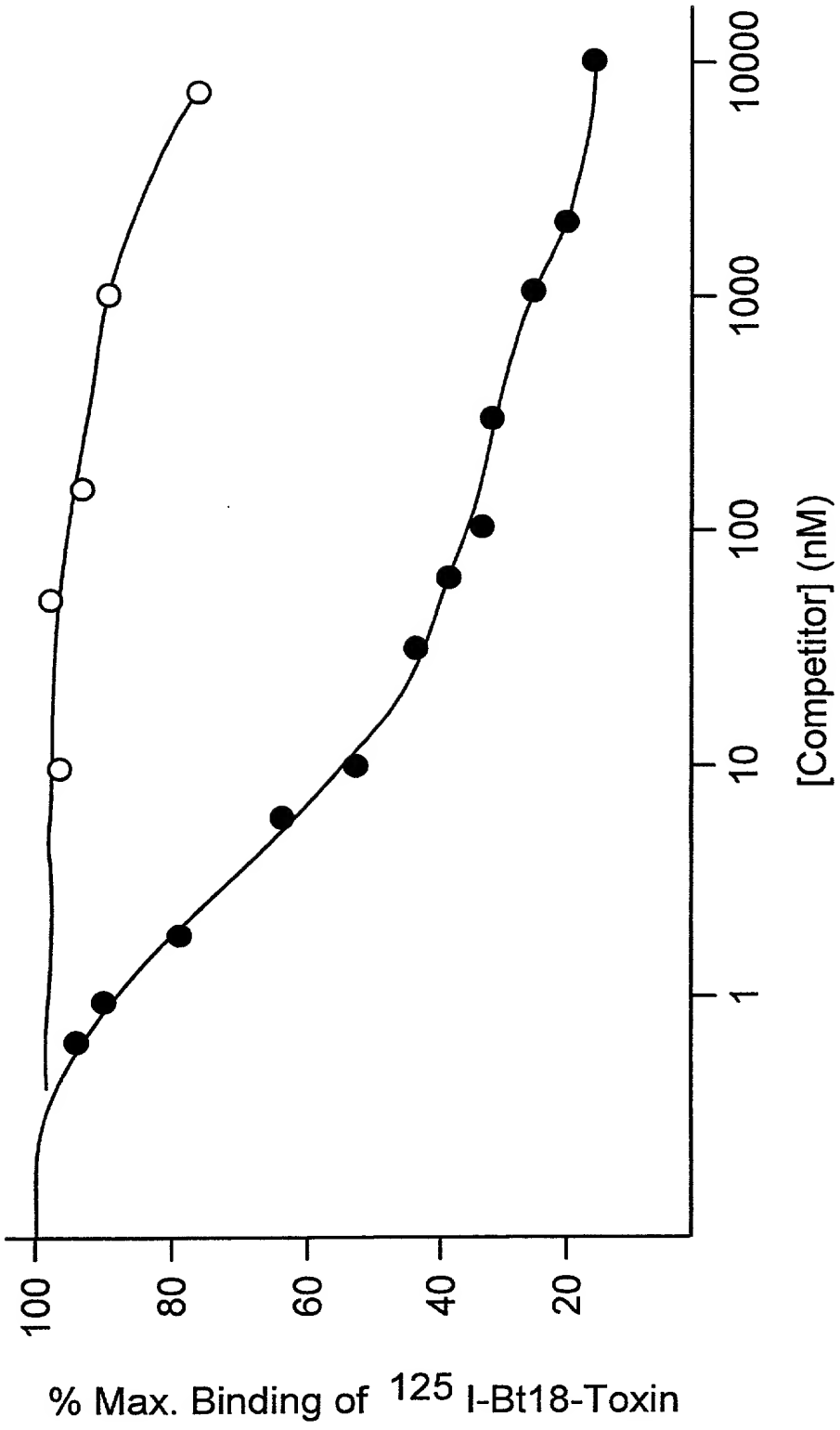

100
80
60
40
20
% Max. Binding of 125 I-Bt18-Toxin
1
10
100
1000
10000
[Competitor] (nM)

FIG. 13A

10	20	30	40	50
GGATCTGTTT	TAATATAAGG	GATTTGTGCC	CTTCTCGTTA	TATTCTTTTA

60	70	80	90	100
TTAGCCCCAA	AAACTAGTGC	AACTAAATAT	TTTTATAATT	ACACTGATTA

110	120	130	140	150
AATACTTTAT	TTTTGGGAGT	AAGATTTATG	CTGAAATGTA	ATAAAATTCG

160	170	180	190	200
TTCCATTTTC	TGTATTTTCT	CATAAAATGT	TTCATATGCT	TTAAATTGTA

210	220	230	240	250
GTAAAGAAAA	ACAGTACAAA	CTTAAAAGGA	CTTTAGTAAT	TTAATAAAAA

260			269			278			287	
AAGGGGATAG	TTT	ATG	GAA	ATA	AAT	AAT	CAA	AAC	CAA	TGT
		MET	Glu	Ile	Asn	Asn	Gln	Asn	Gln	Cys

FIG. 13B

296		305		314		323						
GTG	CCT	TAC	AAT	TGT	TTA	AGT	AAT	CCT	AAG	GAG	ATA	ATA
Val	Pro	Tyr	Asn	Cys	Leu	Ser	Asn	Pro	Lys	Glu	Ile	Ile

332			341			350			359			368
TTA	GGC	GAG	GAA	AGG	CTA	GAA	ACA	GGG	AAT	ACT	GTA	GCA
Leu	Gly	Glu	Glu	Arg	Leu	Glu	Thr	Gly	Asn	Thr	Val	Ala

		377			386			395			404	
GAC	ATT	TCA	TTA	GGG	CTT	ATT	AAT	TTT	CTA	TAT	TCT	AAT
Asp	Ile	Ser	Leu	Gly	Leu	Ile	Asn	Phe	Leu	Tyr	Ser	Asn

	413			422			431			440		
TTT	GTA	CCA	GGA	GGA	GGA	TTT	ATA	GTA	GGT	TTA	CTA	GAA
Phe	Val	Pro	Gly	Gly	Gly	Phe	Ile	Val	Gly	Leu	Leu	Glu

449			458			467			476			485
TTA	ATA	TGG	GGA	TTT	ATA	GGG	CCT	TCG	CAA	TGG	GAT	ATT
Leu	Ile	Trp	Gly	Phe	Ile	Gly	Pro	Ser	Gln	Trp	Asp	Ile

		494			503			512			521	
TTT	TTA	GCT	CAA	ATT	GAG	CAA	TTG	ATT	AGT	CAA	AGA	ATA
Phe	Leu	Ala	Gln	Ile	Glu	Gln	Leu	Ile	Ser	Gln	Arg	Ile

FIG. 13C

	530			539			548			557		
GAA	GAA	TTT	GCT	AGG	AAT	CAG	GCA	ATT	TCA	AGA	TTG	GAG
Glu	Glu	Phe	Ala	Arg	Asn	Gln	Ala	Ile	Ser	Arg	Leu	Glu
566			575			584			593			602
GGG	CTA	AGC	AAT	CTT	TAT	AAG	GTC	TAT	GTT	AGA	GCG	TTT
Gly	Leu	Ser	Asn	Leu	Tyr	Lys	Val	Tyr	Val	Arg	Ala	Phe
		611			620			629			638	
AGC	GAC	TGG	GAG	AAA	GAT	CCT	ACT	AAT	CCT	GCT	TTA	AGG
Ser	Asp	Trp	Glu	Lys	Asp	Pro	Thr	Asn	Pro	Ala	Leu	Arg
	647			656			665			674		
GAA	GAA	ATG	CGT	ATA	CAA	TTT	AAT	GAC	ATG	AAT	AGT	GCT
Glu	Glu	MET	Arg	Ile	Gln	Phe	Asn	Asp	MET	Asn	Ser	Ala
683			692			701			710			719
CTC	ATA	ACG	GCT	ATT	CCA	CTT	TTT	AGA	GTT	CAA	AAT	TAT
Leu	Ile	Thr	Ala	Ile	Pro	Leu	Phe	Arg	Val	Gln	Asn	Tyr
		728			737			746			755	
GAA	GTT	GCT	CTT	TTA	TCT	GTA	TAT	GTT	CAA	GCC	GCA	AAC
Glu	Val	Ala	Leu	Leu	Ser	Val	Tyr	Val	Gln	Ala	Ala	Asn

FIG. 13D

	764			773			782			791		
TTA	CAT	TTA	TCT	ATT	TTA	AGG	GAT	GTT	TCA	GTT	TTC	GGA
Leu	His	Leu	Ser	Ile	Leu	Arg	Asp	Val	Ser	Val	Phe	Gly

800			809			818			827			836
GAA	AGA	TGG	GGA	TAT	GAT	ACA	GCG	ACT	ATC	AAT	AAT	CGC
Glu	Arg	Trp	Gly	Tyr	Asp	Thr	Ala	Thr	Ile	Asn	Asn	Arg

		845			854			863			872	
TAT	AGT	GAT	CTG	ACT	AGC	CTT	ATT	CAT	GTT	TAT	ACT	AAC
Tyr	Ser	Asp	Leu	Thr	Ser	Leu	Ile	His	Val	Tyr	Thr	Asn

	881			890			899			908		
CAT	TGT	GTG	GAT	ACG	TAT	AAT	CAG	GGA	TTA	AGG	CGT	TTG
His	Cys	Val	Asp	Thr	Tyr	Asn	Gln	Gly	Leu	Arg	Arg	Leu

917			926			935			944			953
GAA	GGT	CGT	TTT	CTT	AGC	GAT	TGG	ATT	GTA	TAT	AAT	CGT
Glu	Gly	Arg	Phe	Leu	Ser	Asp	Trp	Ile	Val	Tyr	Asn	Arg

		962			971			980			989	
TTC	CGG	AGA	CAA	TTG	ACA	ATT	TCA	GTA	TTA	GAT	ATT	GTT
Phe	Arg	Arg	Gln	Leu	Thr	Ile	Ser	Val	Leu	Asp	Ile	Val

FIG. 13E

```
      998                1007                1016                1025
GCG TTT TTT CCA AAT TAT GAT ATT AGA ACA TAT CCA ATT
Ala Phe Phe Pro Asn Tyr Asp Ile Arg Thr Tyr Pro Ile

1034                1043                1052                1061                1070
CAA ACA GCT ACT CAG CTA ACG AGG GAA GTC TAT CTG GAT
Gln Thr Ala Thr Gln Leu Thr Arg Glu Val Tyr Leu Asp

           1079                1088                1097                1106
TTA CCT TTT ATT AAT CAA AAT CTT TCT CCT GCA GCA AGC
Leu Pro Phe Ile Asn Glu Asn Leu Ser Pro Ala Ala Ser

      1115                1124                1133                1142
TAT CCA ACC TTT TCA GCT GCT GAA AGT GCT ATA ATT AGA
Tyr Pro Thr Phe Ser Ala Ala Glu Ser Ala Ile Ile Arg

1151                1160                1169                1178                1187
AGT CCT CAT TTA GTA GAC TTT TTA AAT AGC TTT ACC ATT
Ser Pro His Leu Val Asp Phe Leu Asn Ser Phe Thr Ile

           1196                1205                1214                1223
TAT ACA GAT AGT CTG GCA CGT TAT GCA TAT TGG GGA GGG
Tyr Thr Asp Ser Leu Ala Arg Tyr Ala Tyr Trp Gly Gly
```

FIG. 13F

```
      1232          1241          1250          1259
CAC TTG GTA AAT TCT TTC CGC ACA GGA ACC ACT ACT AAT
His Leu Val Asn Ser Phe Arg Thr Gly Thr Thr Thr Asn

1268          1277          1286          1295          1304
TTG ATA AGA TCC CCT TTA TAT GGA AGG GAA GGA AAT ACA
Leu Ile Arg Ser Pro Leu Tyr Gly Arg Glu Gly Asn Thr

      1313          1322          1331          1340
GAG CGC CCC GTA ACT ATT ACC GCA TCA CCT AGC GTA CCA
Glu Arg Pro Val Thr Ile Thr Ala Ser Pro Ser Val Pro

      1349          1358          1367          1376
ATA TTT AGA ACA CTT TCA TAT ATT ACA GGC CTT GAC AAT
Ile Phe Arg Thr Leu Ser Tyr Ile Thr Gly Leu Asp Asn

1385          1394          1403          1412          1421
TCA AAT CCT GTA GCT GGA ATC GAG GGA GTG GAA TTC CAA
Ser Asn Pro Val Ala Gly Ile Glu Gly Val Glu Phe Gln

      1430          1439          1448          1457
AAT ACT ATA AGT AGA AGT ATC TAT CGT AAA AGC GGT CCA
Asn Thr Ile Ser Arg Ser Ile Tyr Arg Lys Ser Gly Pro
```

FIG. 13G

	1466			1475			1484			1493		
ATA	GAT	TCT	TTT	AGT	GAA	TTA	CCA	CCT	CAA	GAT	GCC	AGC
Ile	Asp	Ser	Phe	Ser	Glu	Leu	Pro	Pro	Gln	Asp	Ala	Ser

1502			1511			1520			1529			1538
GTA	TCT	CCT	GCA	ATT	GGG	TAT	AGT	CAC	CGT	TTA	TGC	CAT
Val	Ser	Pro	Ala	Ile	Gly	Tyr	Ser	His	Arg	Leu	Cys	His

		1547			1556			1565			1574	
GCA	ACA	TTT	TTA	GAA	CGG	ATT	AGT	GGA	CCA	AGA	ATA	GCA
Ala	Thr	Phe	Leu	Glu	Arg	Ile	Ser	Gly	Pro	Arg	Ile	Ala

	1583			1592			1601			1610		
GGC	ACC	GTA	TTT	TCT	TGG	ACA	CAC	CGT	AGT	GCC	AGC	CCT
Gly	Thr	Val	Phe	Ser	Trp	Thr	His	Arg	Ser	Ala	Ser	Pro

1619			1628			1637			1646			1655
ACT	AAT	GAA	GTA	AGT	CCA	TCT	AGA	ATT	ACA	CAA	ATT	CCA
Thr	Asn	Glu	Val	Ser	Pro	Ser	Arg	Ile	Thr	Gln	Ile	Pro

		1664			1673			1682			1691	
TGG	GTA	AAG	GCG	CAT	ACT	CTT	GCA	TCT	GGT	GCC	TCC	GTC
Trp	Val	Lys	Ala	His	Thr	Leu	Ala	Ser	Gly	Ala	Ser	Val

FIG. 13H

```
      1700          1709          1718          1727
ATT AAA GGT CCT GGA TTT ACA GGT GGA GAT ATT CTG ACT
Ile Lys Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Thr

1736          1745          1754          1763          1772
AGG AAT AGT ATG GGC GAG CTG GGG ACC TTA CGA GTA ACC
Arg Asn Ser MET Gly Glu Leu Gly Thr Leu Arg Val Thr

      1781          1790          1799          1808
TTC ACA GGA AGA TTA CCA CAA AGT TAT TAT ATA CGT TTC
Phe Thr Gly Arg Leu Pro Gln Ser Tyr Tyr Ile Arg Phe

      1817          1826          1835          1844
CGT TAT GCT TCG GTA GCA AAT AGG AGT GGT ACA TTT AGA
Arg Tyr Ala Ser Val Ala Asn Arg Ser Gly Thr Phe Arg

1853          1862          1871          1880          1889
TAT TCA CAG CCA CCT TCG TAT GGA ATT TCA TTT CCA AAA
Tyr Ser Gln Pro Pro Ser Tyr Gly Ile Ser Phe Pro Lys

      1898          1907          1916          1925
ACT ATG GAC GCA GGT GAA CCA CTA ACA TCT CGT TCG TTC
Thr MET Asp Ala Gly Glu Pro Leu Thr Ser Arg Ser Phe
```

FIG. 13I

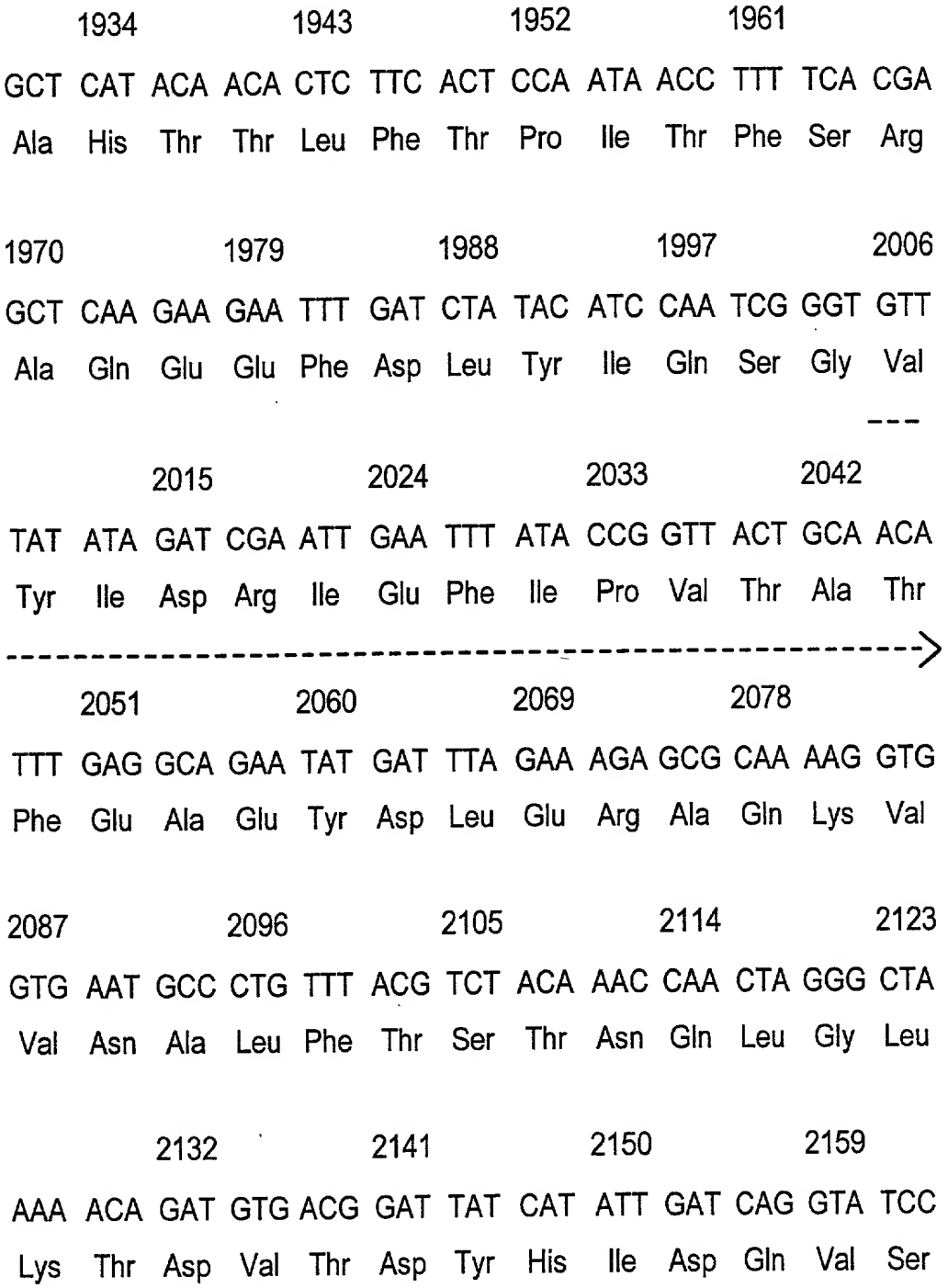

1934 1943 1952 1961
GCT CAT ACA ACA CTC TTC ACT CCA ATA ACC TTT TCA CGA
Ala His Thr Thr Leu Phe Thr Pro Ile Thr Phe Ser Arg
1970 1979 1988 1997 2006
GCT CAA GAA GAA TTT GAT CTA TAC ATC CAA TCG GGT GTT
Ala Gln Glu Glu Phe Asp Leu Tyr Ile Gln Ser Gly Val
2015 2024 2033 2042
TAT ATA GAT CGA ATT GAA TTT ATA CCG GTT ACT GCA ACA
Tyr Ile Asp Arg Ile Glu Phe Ile Pro Val Thr Ala Thr
2051 2060 2069 2078
TTT GAG GCA GAA TAT GAT TTA GAA AGA GCG CAA AAG GTG
Phe Glu Ala Glu Tyr Asp Leu Glu Arg Ala Gln Lys Val
2087 2096 2105 2114 2123
GTG AAT GCC CTG TTT ACG TCT ACA AAC CAA CTA GGG CTA
Val Asn Ala Leu Phe Thr Ser Thr Asn Gln Leu Gly Leu
2132 2141 2150 2159
AAA ACA GAT GTG ACG GAT TAT CAT ATT GAT CAG GTA TCC
Lys Thr Asp Val Thr Asp Tyr His Ile Asp Gln Val Ser

FIG. 13J

2168 2177 2186 2195

AAT CTA GTT GCG TGT TTA TCG GAT GAA TTT TGT CTG GAT

Asn Leu Val Ala Cys Leu Ser Asp Glu Phe Cys Leu Asp

2204 2213 2222 2231 2240

GAA AAG AGA GAA TTG TCC GAG AAA GTT AAA CAT GCA AAG

Glu Lys Arg Glu Leu Ser Glu Lys Val Lys His Ala Lys

2249 2258 2267 2276

CGA CTC AGT GAT GAG CGG AAT TTA CTT CAA GAT CCA AAC

Arg Leu Ser Asp Glu Arg Asn Leu Leu Gln Asp Pro Asn

2285 2294 2303 2312

TTC AGA GGG ATC AAT AGG CAA CCA GAC CGT GGC TGG AGA

Phe Arg Gly Ile Asn Arg Gln Pro Asp Arg Gly Trp Arg

2321 2330 2339 2348 2357

GGA AGT ACG GAT ATT ACT ATC CAA GGA GGA GAT GAC GTA

Gly Ser Thr Asp Ile Thr Ile Gln Gly Gly Asp Asp Val

2366 2375 2384 2393

TTC AAA GAG AAT TAC GTT ACG CTA CCG GGT ACC TTT GAT

Phe Lys Glu Asn Tyr Val Thr Leu Pro Gly Thr Phe Asp

FIG. 13K

	2402			2411			2420			2429		
GAG	TGC	TAT	CCA	ACG	TAT	TTA	TAT	CAA	AAA	ATA	GAT	GAG
Glu	Cys	Tyr	Pro	Thr	Tyr	Leu	Tyr	Gln	Lys	Ile	Asp	Glu

2438			2447			2456			2465			2474
TCG	AAA	TTA	AAA	GCC	TAT	ACC	CGT	TAT	CAA	TTA	AGA	GGG
Ser	Lys	Leu	Lys	Ala	Tyr	Thr	Arg	Tyr	Gln	Leu	Arg	Gly

		2483			2492			2501			2510	
TAT	ATC	GAA	GAT	AGT	CAA	GAC	TTA	GAA	ATC	TAT	TTA	ATT
Tyr	Ile	Glu	Asp	Ser	Gln	Asp	Leu	Glu	Ile	Tyr	Leu	Ile

	2519			2528			2537			2546		
CGT	TAC	AAT	GCA	AAA	CAC	GAA	ATA	GTA	AAT	GTA	CCA	GGT
Arg	Tyr	Asn	Ala	Lys	His	Glu	Ile	Val	Asn	Val	Pro	Gly

2555			2564			2573			2582			2591
ACA	GGA	AGT	TTA	TGG	CCT	CTT	TCT	GTA	GAA	AAT	CAA	ATT
Thr	Gly	Ser	Leu	Trp	Pro	Leu	Ser	Val	Glu	Asn	Gln	Ile

		2600			2609			2618			2627	
GGA	CCT	TGT	GGA	GAA	CCG	AAT	CGA	TGC	GCG	CCA	CAC	CTT
Gly	Pro	Cys	Gly	Glu	Pro	Asn	Arg	Cys	Ala	Pro	His	Leu

FIG. 13L

```
      2636          2645          2654          2663
GAA TGG AAT CCT GAT TTA CAC TGT TCC TGC AGA GAC GGG
Glu Trp Asn Pro Asp Leu His Cys Ser Cys Arg Asp Gly

2672          2681          2690          2699          2708
GAA AAA TGT GCA CAT CAT TCT CAT CAT TTC TCT TTG GAC
Glu Lys Cys Ala His His Ser His His Phe Ser Leu Asp

      2717          2726          2735          2744
ATT GAT GTT GGA TGT ACA GAC TTA AAT GAG GAC TTA GGT
Ile Asp Val Gly Cys Thr Asp Leu Asn Glu Asp Leu Gly

      2753          2762          2771          2780
GTA TGG GTG ATA TTC AAG ATT AAG ACG CAA GAT GGC CAC
Val Trp Val Ile Phe Lys Ile Lys Thr Gln Asp Gly His

2789          2798          2807          2816          2825
GCA CGA CTA GGG AAT CTA GAG TTT CTC GAA GAG AAA CCA
Ala Arg Leu Gly Asn Leu Glu Phe Leu Glu Glu Lys Pro

      2834          2843          2852          2861
TTA TTA GGA GAA GCA CTA GCT CGT GTG AAA AGA GCG GAG
Leu Leu Gly Glu Ala Leu Ala Arg Val Lys Arg Ala Glu
```

FIG. 13M

```
      2870          2879          2888          2897
AAA AAA TGG AGA GAC AAA CGC GAA ACA TTA CAA TTG GAA
Lys Lys Trp Arg Asp Lys Arg Glu Thr Leu Gln Leu Glu

2906          2915          2924          2933          2942
ACA ACT ATC GTT TAT AAA GAG GCA AAA GAA TCT GTA GAT
Thr Thr Ile Val Tyr Lys Glu Ala Lys Glu Ser Val Asp

      2951          2960          2969          2978
GCT TTA TTT GTA AAC TCT CAA TAT GAT AGA TTA CAA GCG
Ala Leu Phe Val Asn Ser Gln Tyr Asp Arg Leu Gln Ala

      2987          2996          3005          3014
GAT ACG AAC ATC GCG ATG ATT CAT GCG GCA GAT AAA CGC
Asp Thr Ash Ile Ala MET Ile His Ala Ala Asp Lys Arg

3023          3032          3041          3050          3059
GTT CAT AGA ATT CGA GAA GCG TAT CTG CCG GAG CTG TCT
Val His Arg Ile Arg Glu Ala Tyr Leu Pro Glu Leu Ser

      3068          3077          3086          3095
GTG ATT CCG GGT GTC AAT GCG GCT ATT TTT GAA GAA TTA
Val Ile Pro Gly Val Asn Ala Ala Ile Phe Glu Glu Leu
```

FIG. 13N

3104 3113 3122 3131

GAA GAG CGT ATT TTC ACT GCA TTT TCC CTA TAT GAT GCG

Glu Glu Arg Ile Phe Thr Ala Phe Ser Leu Tyr Asp Ala

3140 3149 3158 3167 3176

AGA AAT ATT ATT AAA AAT GGC GAT TTC AAT AAT GGC TTA

Arg Asn Ile Ile Lys Asn Gly Asp Phe Asn Asn Gly Leu

3185 3194 3203 3212

TTA TGC TGG AAC GTG AAA GGG CAT GTA GAG GTA GAA GAA

Leu Cys Trp Asn Val Lys Gly His Val Glu Val Glu Glu

3221 3230 3239 3248

CAA AAC AAT CAC CGT TCA GTC CTG GTT ATC CCA GAA TGG

Gln Asn Asn His Arg Ser Val Leu Val Ile Pro Glu Trp

3257 3266 3275 3284 3293

GAG GCA GAA GTG TCA CAA GAG GTT CGT GTC TGT CCA GGT

Glu Ala Glu Val Ser Gln Glu Val Arg Val Cys Pro Gly

3302 3311 3320 3329

CGT GGC TAT ATC CTT CGT GTT ACA GCG TAC AAA GAG GGA

Arg Gly Tyr Ile Leu Arg Val Thr Ala Tyr Lys Glu Gly

FIG. 13P

3338 3347 3356 3365

TAT GGA GAA GGT TGC GTA ACG ATC CAT GAG ATC GAG AAC

Tyr Gly Glu Gly Cys Val Thr Ile His Glu Ile Glu Asn

3374 3383 3392 3401 3410

AAT ACA GAC GAA CTG AAA TTC AAC AAC TGT GTA GAA GAG

Asn Thr Asp Glu Leu Lys Phe Asn Asn Cys Val Glu Glu

3419 3428 3437 3446

GAA GTA TAT CCA AAC AAC ACG GTA ACG TGT ATT AAT TAT

Glu Val Tyr Pro Asn Asn Thr Val Thr Cys Ile Asn Tyr

3455 3464 3473 3482

ACT GCG ACT CAA GAA GAA TAT GAG GGT ACG TAC ACT TCT

Thr Ala Thr Gln Glu Glu Tyr Glu Gly Thr Tyr Thr Ser

3491 3500 3509 3518 3527

CGT AAT CGA GGA TAT GAC GAA GCC TAT GGT AAT AAC CCT

Arg Asn Arg Gly Tyr Asp Glu Ala Tyr Gly Asn Asn Pro

3536 3545 3554 3563

TCC GTA CCA GCT GAT TAT GCG TCA GTC TAT GAA GAA AAA

Ser Val Pro Ala Asp Tyr Ala Ser Val Tyr Glu Glu Lys

FIG. 13Q

3572 3581 3590 3599

TCG TAT ACA GAT AGA CGA AGA GAG AAT CCT TGT GAA TCT

Ser Tyr Thr Asp Arg Arg Arg Glu Asn Pro Cys Glu Ser

3608 3617 3626 3635 3644

AAC AGA GGA TAT GGA GAT TAC ACA CCA CTA CCA GCT GGT

Asn Arg Gly Tyr Gly Asp Tyr Thr Pro Leu Pro Ala Gly

3653 3662 3671 3680

TAT GTA ACA AAG GAA TTA GAG TAC TTC CCA GAG ACC GAT

Tyr Val Thr Lys Glu Leu Glu Tyr Phe Pro Glu Thr Asp

3689 3698 3707 3716

AAG GTA TGG ATT GAG ATT GGA GAA ACA GAA GGA ACA TTC

Lys Val Trp Ile Glu Ile Gly Glu Thr Glu Gly Thr Phe

3725 3734 3743 3752 3761

ATC GTG GAC AGC GTG GAA TTA CTC CTT ATG GAG GAA TAG

Ile Val Asp Ser Val Glu Leu Leu Leu MET Glu Glu •

FIG. 13R

3771	3781	3791	3801	3811
GACCATCCGA	GTATAGCAGT	TTAATAAATA	TTAATTAAAA	TAGTAGTCTA
3821	**3831**	**3841**	**3851**	**3861**
ACTTCCGTTC	CAATTAAATA	AGTAAATTAC	AGTTGTAAAA	AAAAACGAAC
3871	**3881**	**3891**	**3901**	
ATTACTCTTC	AAAGAGCGAT	GTCCGTTTTT	TATATGGTGT	GT

FIG. 14A

10 20 30 40 50

AATAGAATCT CAAATCTCGA TGACTGCTTA GTCTTTTTAA TACTGTCTAC

60 70 80 90 100

TTGACAGGGG TAGGAACATA ATCGGTCAAT TTTAAATATG GGGCATATAT

110 120 130 140 150

TGATATTTTA TAAAATTTGT TACGTTTTTT GTATTTTTTC ATAAGATGTG

160 170 180 190 200

TCATATGTAT TAAATCGTGG TAATGAAAAA CAGTATCAAA CTATCAGAAC

210 220 230 239

TTTGGTAGTT TAATAAAAAA ACGGAGGTAT TTT ATG GAG GAA

----- MET Glu Glu

248		257			266			275				
AAT	AAT	CAA	AAT	CAA	TGC	ATA	CCT	TAC	AAT	TGT	TTA	AGT
Asn	Asn	Gln	Asn	Gln	Cys	Ile	Pro	Tyr	Asn	Cys	Leu	Ser

284			293			302			311			320
AAT	CCT	GAA	GAA	GTA	CTT	TTG	GAT	GGA	GAA	CGG	ATA	TCA
Asn	Pro	Glu	Glu	Val	Leu	Leu	Asp	Gly	Glu	Arg	Ile	Ser

FIG. 14B

		329			338			347			356	
ACT	GGT	AAT	TCA	TCA	ATT	GAT	ATT	TCT	CTG	TCA	CTT	GTT
Thr	Gly	Asn	Ser	Ser	Ile	Asp	Ile	Ser	Leu	Ser	Leu	Val
	365			374			383			392		
CAG	TTT	ATG	GTA	TCT	AAC	TTT	GTA	CCA	GGG	GGA	GGA	TTT
Gln	Phe	Leu	Val	Ser	Asn	Phe	Val	Pro	Gly	Gly	Gly	Phe
401			410			419			428			437
TTA	GTT	GGA	TTA	ATA	GAT	TTT	GTA	TGG	GGA	ATA	GTT	GGC
Leu	Val	Gly	Leu	Ile	Asp	Phe	Val	Trp	Gly	Ile	Val	Gly
		446			455			464			473	
CCT	TCT	CAA	TGG	GAT	GCA	TTT	CTA	GTA	CAA	ATT	GAA	CAA
Pro	Ser	Gln	Trp	Asp	Ala	Phe	Leu	Val	Gln	Ile	Glu	Gln
	482			491			500			509		
TTA	ATT	AAT	GAA	AGA	ATA	GCT	GAA	TTT	GCT	AGG	AAT	GCT
Leu	Ile	Asn	Glu	Arg	Ile	Ala	Glu	Phe	Ala	Arg	Asn	Ala
518			527			536			545			554
GCT	ATT	GCT	AAT	TTA	GAA	GGA	TTA	GGA	AAC	AAT	TTA	AAT
Ala	Ile	Ala	Asn	Leu	Glu	Gly	Leu	Gly	Asn	Asn	Phe	Asn

FIG. 14C

		563			572			581			590	
ATA	TAT	GTG	GAA	GCA	TTT	AAA	GAA	TGG	GAA	GAA	GAT	CCT
Ile	Tyr	Val	Glu	Ala	Phe	Lys	Glu	Trp	Glu	Glu	Asp	Pro

	599			608			617			626		
AAT	AAT	CCA	GAA	ACC	AGG	ACC	AGA	GTA	ATT	GAT	CGC	TTT
Asn	Asn	Pro	Glu	Thr	Arg	Thr	Arg	Val	Ile	Asp	Arg	Phe

635			644			653			662			671
CGT	ATA	CTT	GAT	GGG	CTA	CTT	GAA	AGG	GAC	ATT	CCT	TCG
Arg	Ile	Leu	Asp	Gly	Leu	Leu	Glu	Arg	Asp	Ile	Pro	Ser

		680			689			698			707	
TTT	CGA	ATT	TCT	GGA	TTT	GAA	GTA	CCC	CTT	TTA	TCC	GTT
Phe	Arg	Ile	Ser	Gly	Phe	Glu	Val	Pro	Leu	Leu	Ser	Val

	716			725			734			743		
TAT	GCT	CAA	GCG	GCC	AAT	CTG	CAT	CTA	GCT	ATA	TTA	AGA
Tyr	Ala	Gln	Ala	Ala	Asn	Leu	His	Leu	Ala	Ile	Leu	Arg

752			761			770			779			788
GAT	TCT	GTA	ATT	TTT	GGA	GAA	AGA	TGG	GGA	TTG	ACA	ACG
Asp	Ser	Val	Ile	Phe	Gly	Glu	Arg	Trp	Gly	Leu	Thr	Thr

FIG. 14D

		797			806			815			824	
ATA	AAT	GTC	AAT	GAA	AAC	TAT	AAT	AGA	CTA	ATT	AGG	CAT
Ile	Asn	Val	Asn	Glu	Asn	Tyr	Asn	Arg	Leu	Ile	Arg	His

	833			842			851			860		
ATT	GAT	GAA	TAT	GCT	GAT	CAC	TGT	GCA	AAT	ACG	TAT	AAT
Ile	Asp	Glu	Tyr	Ala	Asp	His	Cys	Ala	Asn	Thr	Tyr	Asn

869			878			887			896			905
CGG	GGA	TTA	AAT	AAT	TTA	CCG	AAA	TCT	ACG	TAT	CAA	GAT
Arg	Gly	Leu	Asn	Asn	Leu	Pro	Lys	Ser	Thr	Tyr	Gln	Asp

		914			923			932			941	
TGG	ATA	ACA	TAT	AAT	CGA	TTA	CGG	AGA	GAC	TTA	ACA	TTG
Trp	Ile	Thr	Tyr	Asn	Arg	Leu	Arg	Arg	Asp	Leu	Thr	Leu

	950			959			968			977		
ACT	GTA	TTA	GAT	ATC	GCC	GCT	TTC	TTT	CCA	AAC	TAT	GAC
Thr	Val	Leu	Asp	Ile	Ala	Ala	Phe	Phe	Pro	Asn	Tyr	Asp

986			995			1004			1013			1022
AAT	AGG	AGA	TAT	CCA	ATT	CAG	CCA	GTT	GGT	CAA	CTA	ACA
Asn	Arg	Arg	Tyr	Pro	Ile	Gln	Pro	Val	Gly	Gln	Leu	Thr

FIG. 14E

		1031			1040			1049			1058	
AGG	GAA	GTT	TAT	ACG	GAC	CCA	TTA	ATT	AAT	TTT	AAT	CCA
Arg	Glu	Val	Tyr	Thr	Asp	Pro	Leu	Ile	Asn	Phe	Asn	Pro

	1067			1076			1085			1094		
CAG	TTA	CAG	TCT	GTA	GCT	CAA	TTA	CCT	ACT	TTT	AAC	GTT
Gln	Leu	Gln	Ser	Val	Ala	Gln	Leu	Pro	Thr	Phe	Asn	Val

1103			1112			1121			1130			1139
ATG	GAG	AGC	AGC	GCA	ATT	AGA	AAT	CCT	CAT	TTA	TTT	GAT
MET	Glu	Ser	Ser	Ala	Ile	Arg	Asn	Pro	His	Leu	Phe	Asp

		1148			1157			1166			1175	
ATA	TTG	AAT	AAT	CTT	ACA	ATC	TTT	ACG	GAT	TGG	TTT	AGT
Ile	Leu	Asn	Asn	Leu	Thr	Ile	Phe	Thr	Asp	Trp	Phe	Ser

	1184			1193			1202			1211		
GTT	GGA	CGC	AAT	TTT	TAT	TGG	GGA	GGA	CAT	CGA	GTA	ATA
Val	Gly	Arg	Asn	Phe	Tyr	Trp	Gly	Gly	His	Arg	Val	Ile

1220			1229			1238			1247			1256
TCT	AGC	CTT	ATA	GGA	GGT	GGT	AAC	ATA	ACA	TCT	CCT	ATA
Ser	Ser	Leu	Ile	Gly	Gly	Gly	Asn	Ile	Thr	Ser	Pro	Ile

FIG. 14F

		1265			1274			1283			1292	
TAT	GGA	AGA	GAG	GCG	AAC	CAG	GAG	CCT	CCA	AGA	TCC	TTT
Tyr	Gly	Arg	Glu	Ala	Asn	Gln	Glu	Pro	Pro	Arg	Ser	Phe

	1301			1310			1319			1328		
ACT	TTT	AAT	GGA	CCG	GTA	TTT	AGG	ACT	TTA	TCA	AAT	CCT
Thr	Phe	Asn	Gly	Pro	Val	Phe	Arg	Thr	Leu	Ser	Asn	Pro

1337			1346			1355			1364			1373
ACT	TTA	CGA	TTA	TTA	CAG	CAA	CCT	TGG	CCA	GCG	CCA	CCA
Thr	Leu	Arg	Leu	Leu	Gln	Gln	Pro	Trp	Pro	Ala	Pro	Pro

		1382			1391			1400			1409	
TTT	AAT	TTA	CGT	GGT	GTT	GAA	GGA	GTA	GAA	TTT	TCT	ACA
Phe	Ash	Leu	Arg	Gly	Val	Glu	Gly	Val	Glu	Phe	Ser	Thr

	1418			1427			1436			1445		
CCT	ACA	AAT	AGC	TTT	ACG	TAT	CGA	GGA	AGA	GGT	ACG	GTT
Pro	Thr	Asn	Ser	Phe	Thr	Tyr	Arg	Gly	Arg	Gly	Thr	Val

1454			1463			1472			1481			1490
GAT	TCT	TTA	ACT	GAA	TTA	CCG	CCT	GAG	GAT	AAT	AGT	GTG
Asp	Ser	Leu	Thr	Glu	Leu	Pro	Pro	Glu	Asp	Asn	Ser	Val

FIG. 14G

		1499			1508			1517			1526	
CCA	CCT	CGC	GAA	GGA	TAT	AGT	CAT	CGT	TTA	TGT	CAT	GCA
Pro	Pro	Arg	Glu	Gly	Tyr	Ser	His	Arg	Leu	Cys	His	Ala
	1535			1544			1553			1562		
ACT	TTT	GTT	CAA	AGA	TCT	GGA	ACA	CCT	TTT	TTA	ACA	ACT
Thr	Phe	Val	Gln	Arg	Ser	Gly	Thr	Pro	Phe	Leu	Thr	Thr
1571			1580			1589			1598			1607
GGT	GTA	GTA	TTT	TCT	TGG	ACG	CAT	CGT	AGT	GCA	ACT	CTT
Gly	Val	Val	Phe	Ser	Trp	Thr	His	Arg	Ser	Ala	Thr	Leu
		1616			1625			1634			1643	
ACA	AAT	ACA	ATT	GAT	CCA	GAG	AGA	ATT	AAT	CAA	ATA	CCT
Thr	Asn	Thr	Ile	Asp	Pro	Glu	Arg	Ile	Asn	Gln	Ile	Pro
	1652			1661			1670			1679		
TTA	GTG	AAA	GGA	TTT	AGA	GTT	TGG	GGG	GGC	ACC	TCT	GTC
Leu	Val	Lys	Gly	Phe	Arg	Val	Trp	Gly	Gly	Thr	Ser	Val
1688			1697			1706			1715			1724
ATT	ACA	GGA	CCA	GGA	TTT	ACA	GGA	GGG	GAT	ATC	CTT	CGA
Ile	Thr	Gly	Pro	Gly	Phe	Thr	Gly	Gly	Asp	Ile	Leu	Arg

FIG. 14H

		1733			1742			1751			1760	
AGA	AAT	ACC	TTT	GGT	GAT	TTT	GTA	TCT	CTA	CAA	GTC	AAT
Arg	Asn	Thr	Phe	Gly	Asp	Phe	Val	Ser	Leu	Gln	Val	Asn
	1769			1778			1787			1796		
ATT	AAT	TCA	CCA	ATT	ACC	CAA	AGA	TAC	CGT	TTA	AGA	TTT
Ile	Asn	Ser	Pro	Ile	Thr	Gln	Arg	Tyr	Arg	Leu	Arg	Phe
1805			1814			1823			1832			1841
CGT	TAC	GCT	TCC	AGT	AGG	GAT	GCA	CGA	GTT	ATA	GTA	TTA
Arg	Tyr	Ala	Ser	Ser	Arg	Asp	Ala	Arg	Val	Ile	Val	Leu
		1850			1859			1868			1877	
ACA	GGA	GCG	GCA	TCC	ACA	GGA	GTG	GGA	GGC	CAA	GTT	AGT
Thr	Gly	Ala	Ala	Ser	Thr	Gly	Val	Gly	Gly	Gln	Val	Ser
	1886			1895			1904			1913		
GTA	AAT	ATG	CCT	CTT	CAG	AAA	ACT	ATG	GAA	ATA	GGG	GAG
Val	Asn	MET	Pro	Leu	Gln	Lys	Thr	MET	Glu	Ile	Gly	Glu
1922			1931			1940			1949			1958
AAC	TTA	ACA	TCT	AGA	ACA	TTT	AGA	TAT	ACC	GAT	TTT	AGT
Asn	Leu	Thr	Ser	Arg	Thr	Phe	Arg	Tyr	Thr	Asp	Phe	Ser

FIG. 14I

		1967			1976			1985			1994	
AAT	CCT	TTT	TCA	TTT	AGA	GCT	AAT	CCA	GAT	ATA	ATT	GGG
Asn	Pro	Phe	Ser	Phe	Arg	Ala	Asn	Pro	Asp	Ile	Ile	Gly

	2003			2012			2021			2030		
ATA	AGT	GAA	CAA	CCT	CTA	TTT	GGT	GCA	GGT	TCT	ATT	AGT
Ile	Ser	Glu	Gln	Pro	Leu	Phe	Gly	Ala	Gly	Ser	Ile	Ser

2039			2048			2057			2066			2075
AGC	GGT	GAA	CTT	TAT	ATA	GAT	AAA	ATT	GAA	ATT	ATT	CTA
Ser	Gly	Glu	Leu	Tyr	Ile	Asp	Lys	Ile	Glu	Ile	Ile	Leu

		2084			2093			2102			2111	
GCA	GAT	GCA	ACA	TTT	GAA	GCA	GAA	TCT	GAT	TTA	GAA	AGA
Ala	Asp	Ala	Thr	Phe	Glu	Ala	Glu	Ser	Asp	Leu	Glu	Arg

	2120			2129			2138			2147		
GCA	CAA	AAG	GCG	GTG	AAT	GCC	CTG	TTT	ACT	TCT	TCC	AAT
Ala	Gln	Lya	Ala	Val	Asn	Ala	Leu	Phe	Thr	Ser	Ser	Asn

2156			2165			2174			2183			2192
CAA	ATC	GGG	TTA	AAA	ACC	GAT	GTG	ACG	GAT	TAT	CAT	ATT
Gln	Ile	Gly	Leu	Lys	Thr	Asp	Val	Thr	Asp	Tyr	His	Ile

FIG. 14J

```
                2201                2210                2219                2228
GAT CAA GTA TCC AAT TTA GTG GAT TGT TTA TCA GAT GAA
Asp Gln Val Ser Asn Leu Val Asp Cys Leu Ser Asp Glu

     2237                2246                2255                2264
TTT TGT CTG GAT GAA AAG CGA GAA TTG TCC GAG AAA GTC
Phe Cys Leu Asp Glu Lys Arg Glu Leu Ser Glu Lys Val

2273                2282                2291                2300                2309
AAA CAT GCG AAG CGA CTC AGT GAT GAG CGG AAT TTA CTT
Lys His Ala Lys Arg Leu Ser Asp Glu Arg Asn Leu Leu

                2318                2327                2336                2345
CAA GAT CCA AAC TTC AGA GGG ATC AAT AGA CAA CCA GAC
Gln Asp Pro Asn Phe Arg Gly Ile Asn Arg Gln Pro Asp

     2354                2363                2372                2381
CGT GGC TGG AGA GGA AGT ACA GAT ATT ACC ATC CAA GGA
Arg Gly Trp Arg Gly Ser Thr Asp Ile Thr Ile Gln Gly

2390                2399                2408                2417                2426
GGA GAT GAC GTA TTC AAA GAG AAT TAC GTC ACA CTA CCG
Gly Asp Asp Val Phe Lys Glu Asn Tyr Val Thr Leu Pro
```

FIG. 14K

```
          2435          2444          2453          2462
GGT ACC GTT GAT GAG TGC TAT CCA ACG TAT TTA TAT CAG
Gly Thr Val Asp Glu Cys Tyr Pro Thr Tyr Leu Tyr Gln

     2471          2480          2489          2498
AAA ATA GAT GAG TCG AAA TTA AAA GCT TAT ACC CGT TAT
Lys Ile Asp Glu Ser Lys Leu Lys Ala Tyr Thr Arg Tyr

2507          2516          2525          2534          2543
GAA TTA AGA GGG TAT ATC GAA GAT AGT CAA GAC TTA GAA
Glu Leu Arg Gly Tyr Ile Glu Asp Ser Gln Asp Leu Glu

          2552          2561          2570          2579
ATC TAT TTG ATC CGT TAC AAT GCA AAA CAC GAA ATA GTA
Ile Tyr Leu Ile Arg Tyr Asn Ala Lys His Glu Ile Val

     2588          2597          2606          2615
AAT GTG CCA GGC ACG GGT TCC TTA TGG CCG CTT TCA GCC
Asn Val Pro Gly Thr Gly Ser Leu Trp Pro Leu Ser Ala

2624          2633          2642          2651          2660
CAA AGT CCA ATC GGA AAG TGT GGA GAA CCG AAT CGA TGC
Gln Ser Pro Ile Gly Lys Cys Gly Glu Pro Asn Arg Cys
```

FIG. 14L

```
                2669                2678                2687                2696
GCG CCA CAC CTT GAA TGG AAT CCT GAT CTA GAT TGT TCC
Ala Pro His Leu Glu Trp Asn Pro Asp Leu Asp Cys Ser

     2705                2714                2723                2732
TGC AGA GAC GGG GAA AAA TGT GCA CAT CAT TCC CAT CAT
Cys Arg Asp Gly Glu Lys Cys Ala His His Ser His His

2741                2750                2759                2768                2777
TTC ACC TTG GAT ATT GAT GTT GGA TGT ACA GAC TTA AAT
Phe Thr Leu Asp Ile Asp Val Gly Cys Thr Asp Leu Asn

                2786                2795                2804                2813
GAG GAC TTA GGT GTA TGG GTG ATA TTC AAG ATT AAG ACG
Glu Asp Leu Gly Val Trp Val Ile Phe Lys Ile Lys Thr

     2822                2831                2840                2849
CAA GAT GGC CAT GCA AGA CTA GGG AAT CTA GAG TTT CTC
Gln Asp Gly His Ala Arg Leu Gly Asn Leu Glu Phe Leu

2858                2867                2876                2885                2894
GAA GAG AAA CCA TTA TTA GGG GAA GCA CTA GCT CGT GTG
Glu Glu Lys Pro Leu Leu Gly Glu Ala Leu Ala Arg Val
```

FIG. 14M

```
                2903                2912                2921                2930
AAA AGA GCG GAG AAG AAG TGG AGA GAC AAA CGA GAG AAA
Lys Arg Ala Glu Lys Lys Trp Arg Asp Lys Arg Glu Lys

     2939                2948                2957                2966
CTG CAG TTG GAA ACA AAT ATT GTT TAT AAA GAG GCA AAA
Leu Gln Leu Glu Thr Asn Ile Val Tyr Lys Glu Ala Lys

2975                2984                2993                3002                3011
GAA TCT GTA GAT GCT TTA TTT GTA AAC TCT CAA TAT GAT
Glu Ser Val Asp Ala Leu Phe Val Asn Ser Gln Tyr Asp

                3020                3029                3038                3047
AGA TTA CAA GTG GAT ACG AAC ATC GCG ATG ATT CAT GCG
Arg Leu Gln Val Asp Thr Asn Ile Ala MET Ile His Ala

     3056                3065                3074                3083
GCA GAT AAA CGC GTT CAT AGA ATC CGG GAA GCG TAT CTG
Ala Asp Lys Arg Val His Arg Ile Arg Glu Ala Tyr Leu

3092                3101                3110                3119                3128
CCA GAG TTG TCT GTG ATT CCA GGT GTC AAT GCG GCC ATT
Pro Glu Leu Ser Val Ile Pro Gly Val Asn Ala Ala Ile
```

FIG. 14N

3137 3146 3155 3164

TTC GAA GAA TTA GAG GGA CGT ATT TTT ACA GCG TAT TCC

Phe Glu Glu Leu Glu Gly Arg Ile Phe Thr Ala Tyr Ser

3173 3182 3191 3200

TTA TAT GAT GCG AGA AAT GTC ATT AAA AAT GGC GAT TTC

Leu Tyr Asp Ala Arg Asn Val Ile Lys Asn Gly Asp Phe

3209 3218 3227 3236 3245

AAT AAT GGC TTA TTA TGC TGG AAC GTG AAA GGT CAT GTA

Asn Asn Gly Leu Leu Cys Trp Asn Val Lys Gly His Val

3254 3263 3272 3281

GAT GTA GAA GAG CAA AAC AAC CAC CGT TCG GTC CTT GTT

Asp Val Glu Glu Gln Asn Asn His Arg Ser Val Leu Val

3290 3299 3308 3317

ATC CCA GAA TGG GAG GCA GAA GTG TCA CAA GAG GTT CGT

Ile Pro Glu Trp Glu Ala Glu Val Ser Gln Glu Val Arg

3326 3335 3344 3353 3362

GTC TGT CCA GGT CGT GGC TAT ATC CTT CGT GTC ACA GCA

Val Cys Pro Gly Arg Gly Tyr Ile Leu Arg Val Thr Ala

FIG. 14P

		3371			3380			3389			3398	
TAT	AAA	GAG	GGA	TAT	GGA	GAG	GGC	TGC	GTA	ACG	ATC	CAT
Tyr	Lys	Glu	Gly	Tyr	Gly	Glu	Gly	Cys	Val	Thr	Ile	His

	3407			3416			3425			3434		
GAG	ATC	GAA	GAC	AAT	ACA	GAC	GAA	CTG	AAA	TTC	AGC	AAC
Glu	Ile	Glu	Asp	Asn	Thr	Asp	Glu	Leu	Lys	Phe	Ser	Asn

3443			3452			3461			3470			3479
TGT	GTA	GAA	GAG	GAA	GTA	TAT	CCA	AAC	AAC	ACA	GTA	ACG
Cys	Val	Glu	Glu	Glu	Val	Tyr	Pro	Asn	Asn	Thr	Val	Thr

		3488			3497			3506			3515	
TGT	AAT	AAT	TAT	ACT	GGG	ACT	CAA	GAA	GAA	TAT	GAG	GGT
Cys	Asn	Asn	Tyr	Thr	Gly	Thr	Gln	Glu	Glu	Tyr	Glu	Gly

	3524			3533			3542			3551		
ACG	TAC	ACT	TCT	CGT	AAT	CAA	GGA	TAT	GAC	GAA	GCC	TAT
Thr	Tyr	Thr	Ser	Arg	Asn	Gln	Gly	Tyr	Asp	Glu	Ala	Tyr

3560			3569			3578			3587			3596
GGT	AAT	AAC	CCT	TCC	GTA	CCA	GCT	GAT	TAC	GCT	TCA	GTC
Gly	Asn	Asn	Pro	Ser	Val	Pro	Ala	Asp	Tyr	Ala	Ser	Val

FIG. 14Q

```
          3605          3614          3623          3632
TAT GAA GAA AAA TCG TAT ACA GAT GGA CGA AGA GAG AAT
Tyr Glu Glu Lys Ser Tyr Thr Asp Gly Arg Arg Glu Asn

     3641          3650          3659          3668
CCT TGT GAA TCT AAC AGA GGC TAT GGG GAT TAC ACA CCA
Pro Cys Glu Ser Asn Arg Gly Tyr Gly Asp Tyr Thr Pro

3677          3686          3695          3704          3713
CTA CCG GCT GGT TAT GTA ACA AAG GAT TTA GAG TAC TTC
Leu Pro Ala Gly Tyr Val Thr Lys Asp Leu Glu Tyr Phe

          3722          3731          3740          3749
CCA GAG ACC GAT AAG GTA TGG ATT GAG ATC GGA GAA ACA
Pro Glu Thr Asp Lys Val Trp Ile Glu Ile Gly Glu Thr

     3758          3767          3776          3785
GAA GGA ACA TTC ATC GTG GAT AGC GTG GAA TTA CTC CTT
Glu Gly Thr Phe Ile Val Asp Ser Val Glu Leu Leu Leu

3794          3803          3813          3823          3833
ATG GAG GAA TAA GATACGTTAT AAAATGTAAC GTATGCAAAT
MET Glu Glu •
```

FIG. 14R

3843 3853 3863 3873 3883

AAAGAATGAT TACTGACCTA TATTAACAGA TAAATAAGAA AATTTTTATA

3893 3903 3913 3923

CGAATAAAAA ACGGACATCA CTCTTAAGAG AATGATGTCC

FIG. 15A

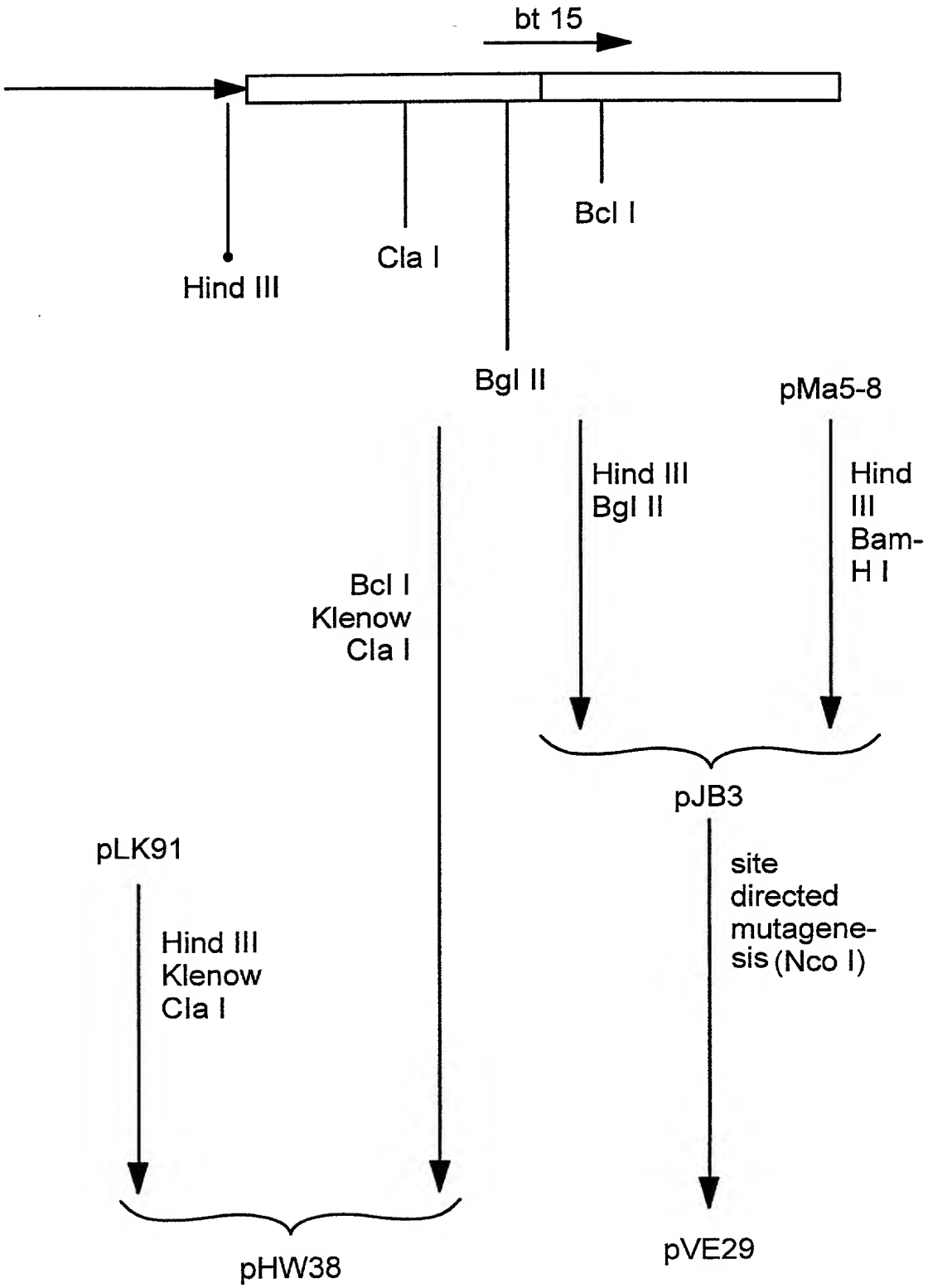

bt 15
Hind III
Cla I
Bcl I
Bgl II
pMa5-8
Hind III
Bgl II
Hind III Bam-H I
Bcl I Klenow Cla I
pJB3
pLK91
Hind III Klenow Cla I
site directed mutagene-sis (Nco I)
pHW38
pVE29

FIG. 15B

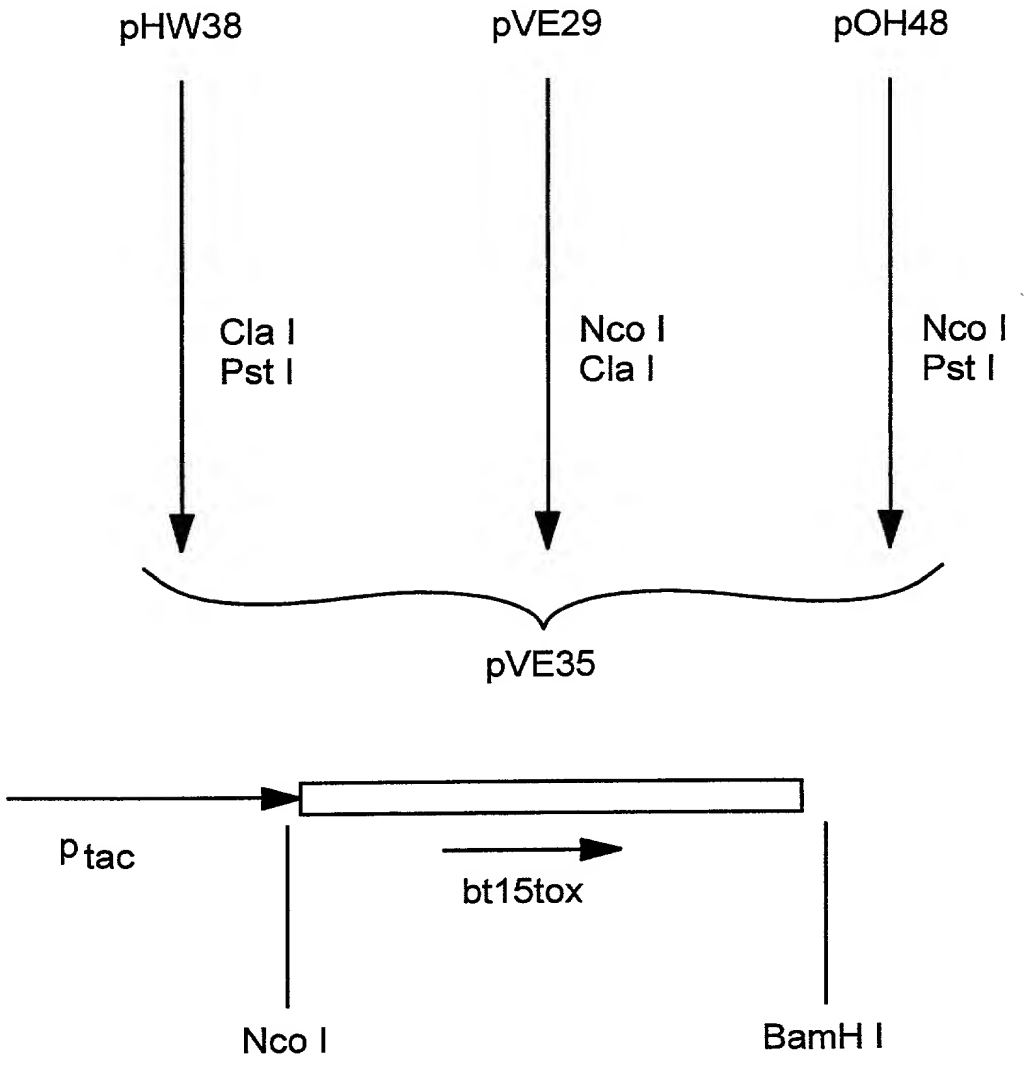

pHW38
pVE29
pOH48
Cla I
Pst I
Nco I
Cla I
Nco I
Pst I
pVE35
p tac
bt15tox
Nco I
BamH I

FIG. 15C

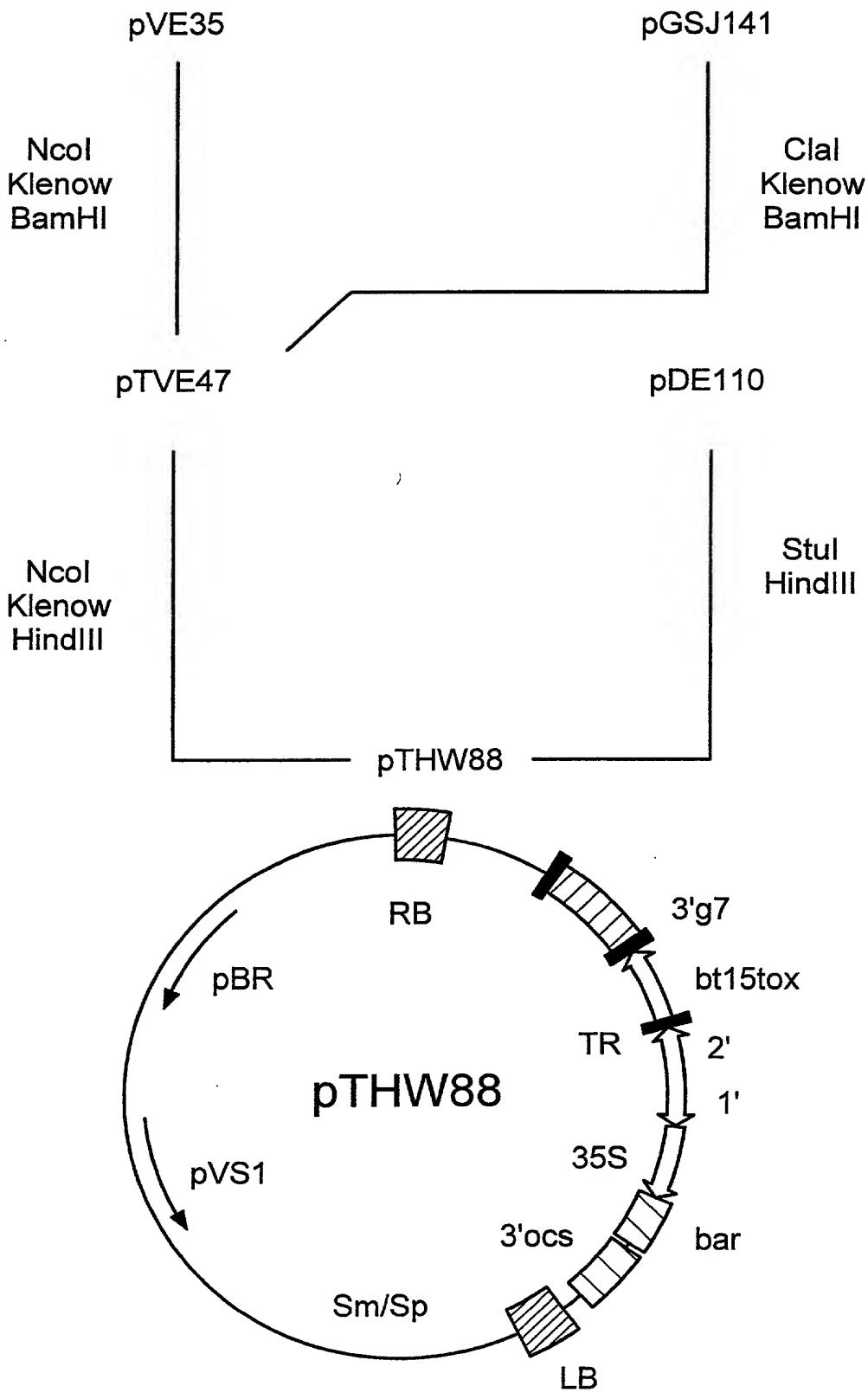

pVE35
pGSJ141
NcoI
Klenow
BamHI
ClaI
Klenow
BamHI
pTVE47
pDE110
NcoI
Klenow
HindIII
StuI
HindIII
pTHW88
RB
3'g7
pBR
bt15tox
TR
2'
pTHW88
1'
35S
pVS1
3'ocs
bar
Sm/Sp
LB

FIG. 16A

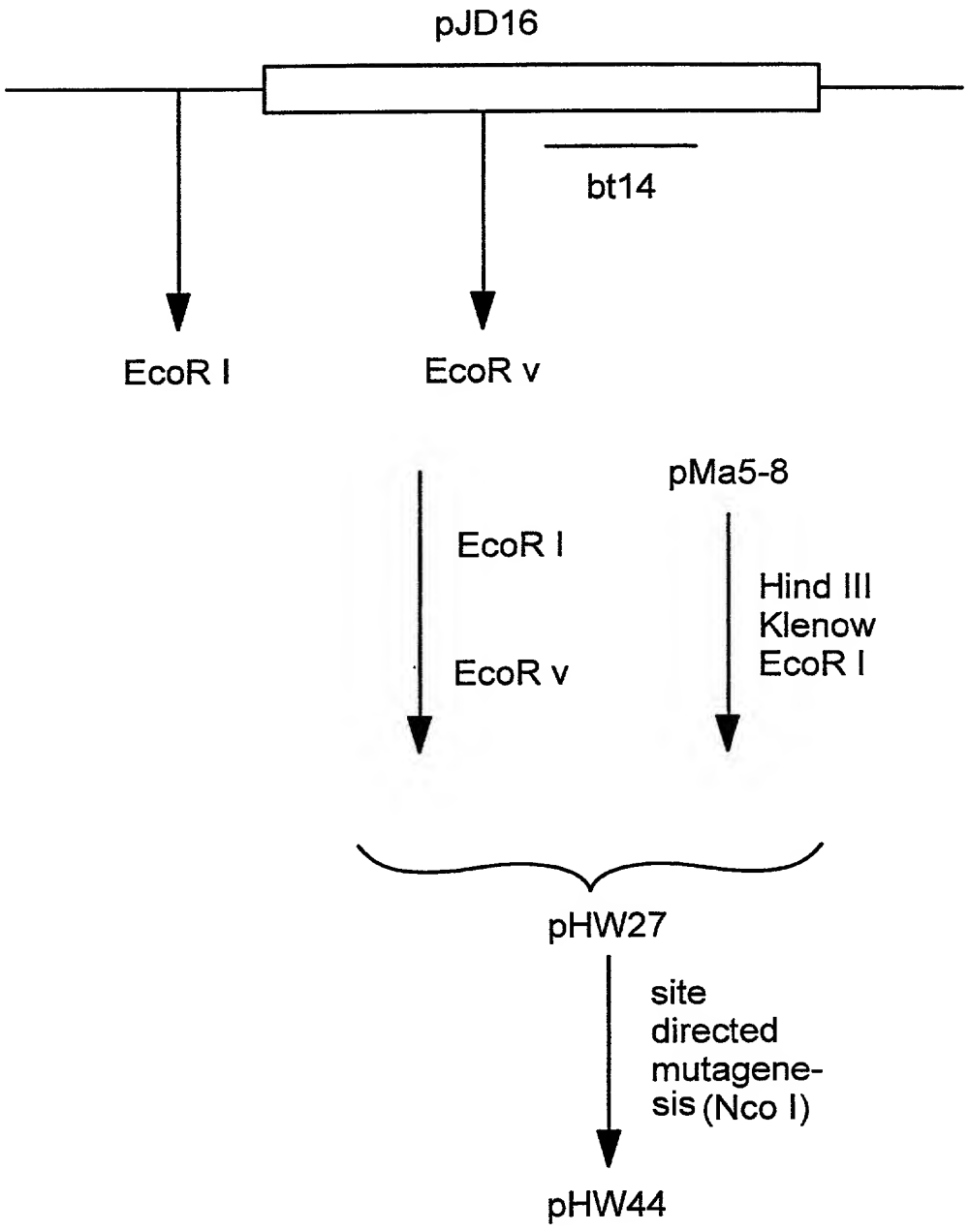
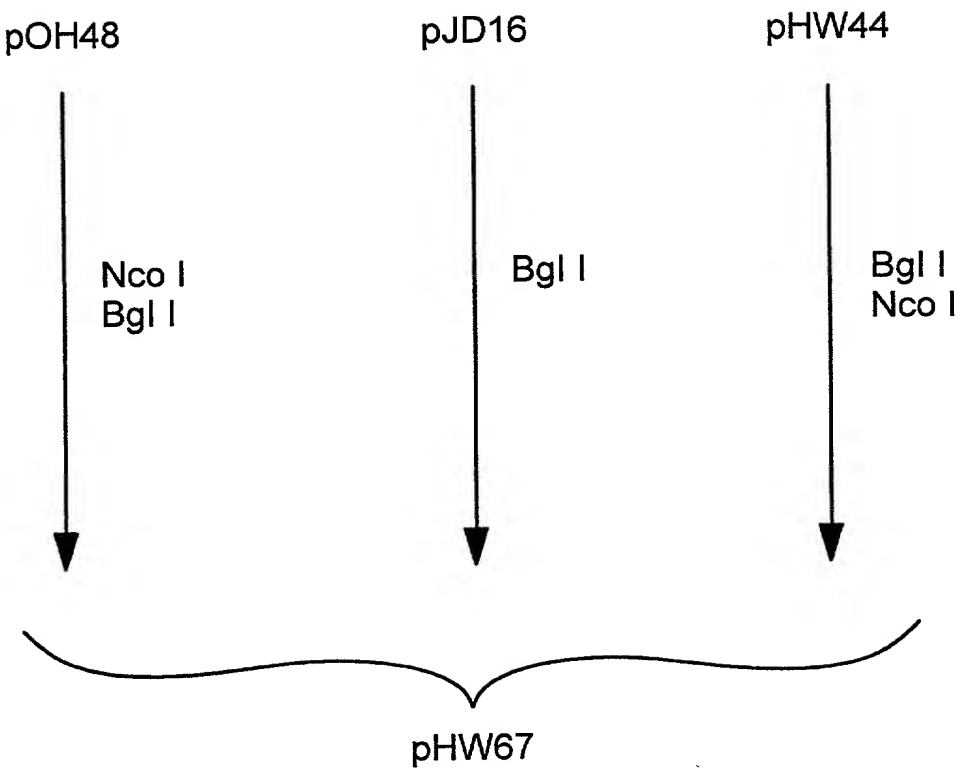

pJD16
bt14
EcoR I
EcoR v
pMa5-8
EcoR I
EcoR v
Hind III
Klenow
EcoR I
pHW27
site
directed
mutagene-
sis (Nco I)
pHW44

FIG. 16B


pOH48
pJD16
pHW44
Nco I
Bgl I
Bgl I
Bgl I
Nco I
pHW67

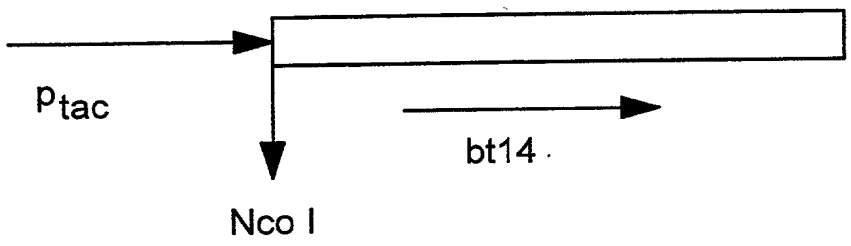

p_{tac}
bt14
Nco I

FIG. 16C

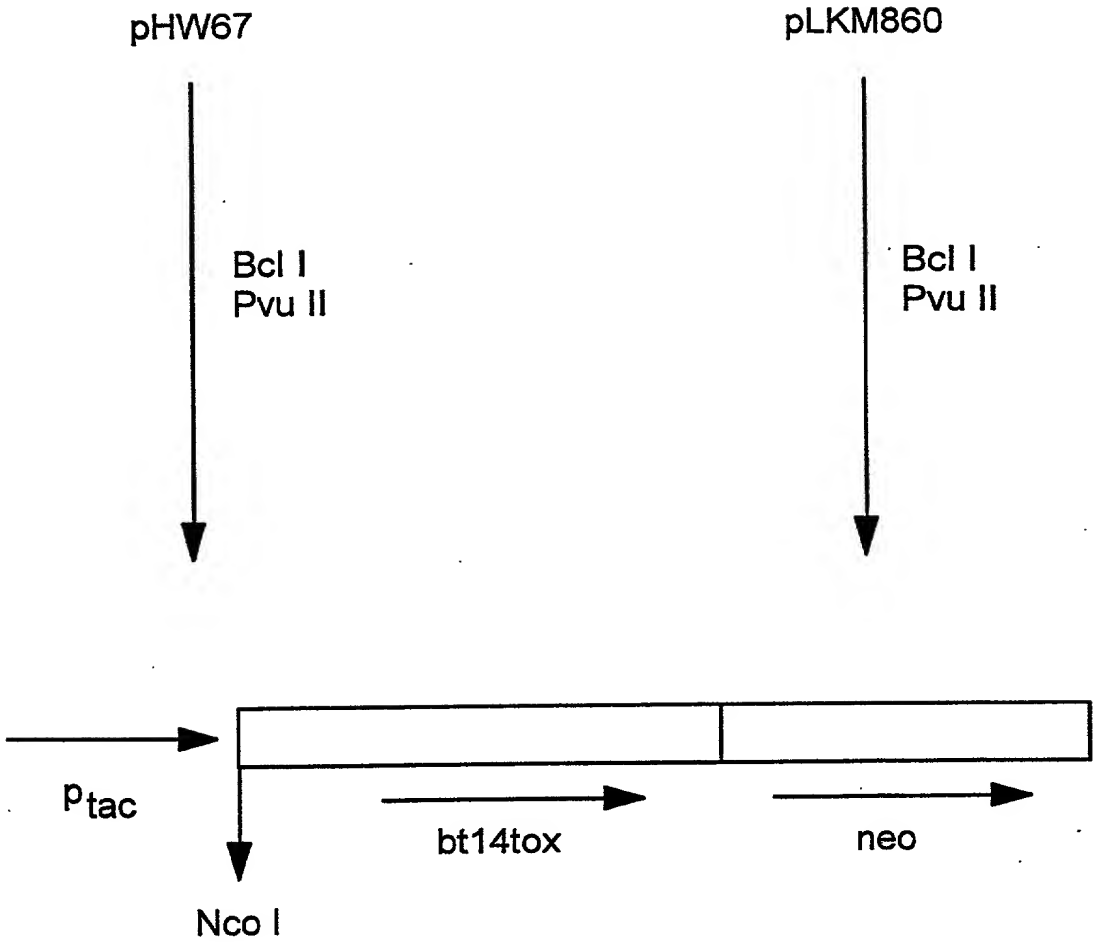


FIG. 16D

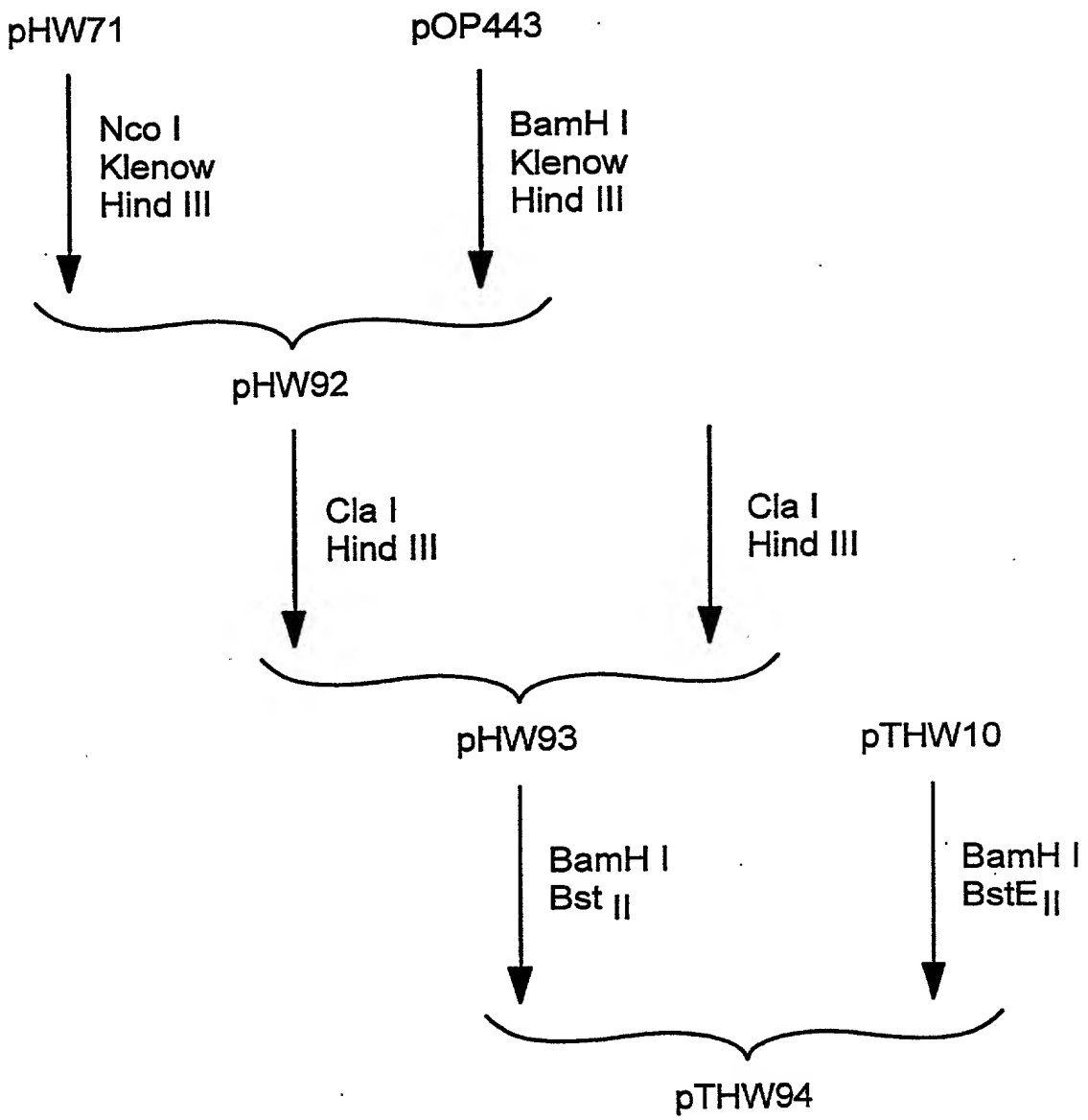
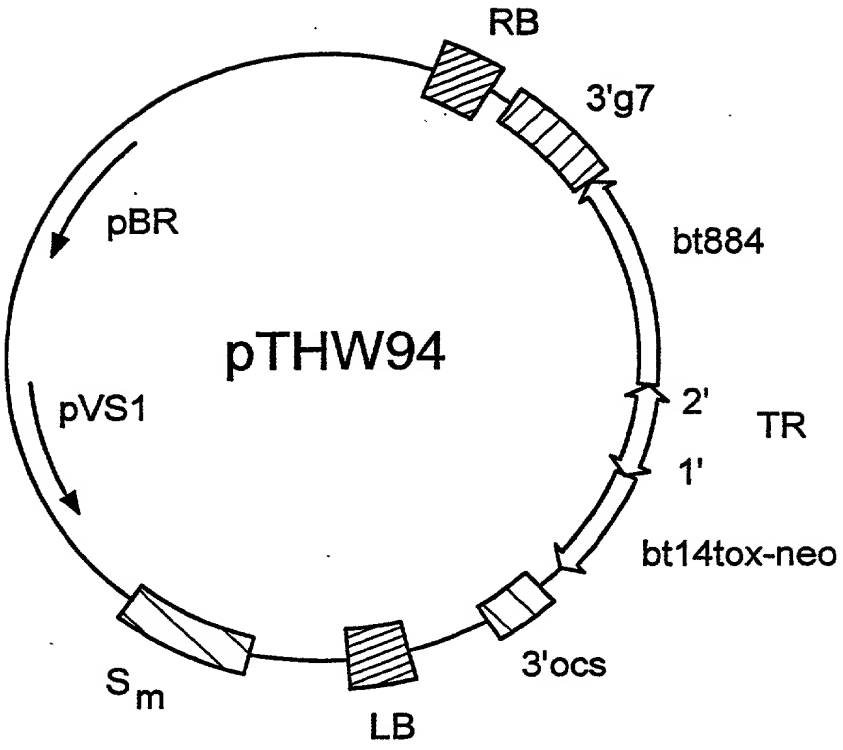

pHW71
pOP443
Nco I
Klenow
Hind III
BamH I
Klenow
Hind III
pHW92
Cla I
Hind III
Cla I
Hind III
pHW93
pTHW10
BamH I
Bst II
BamH I
BstE II
pTHW94

FIG. 16E



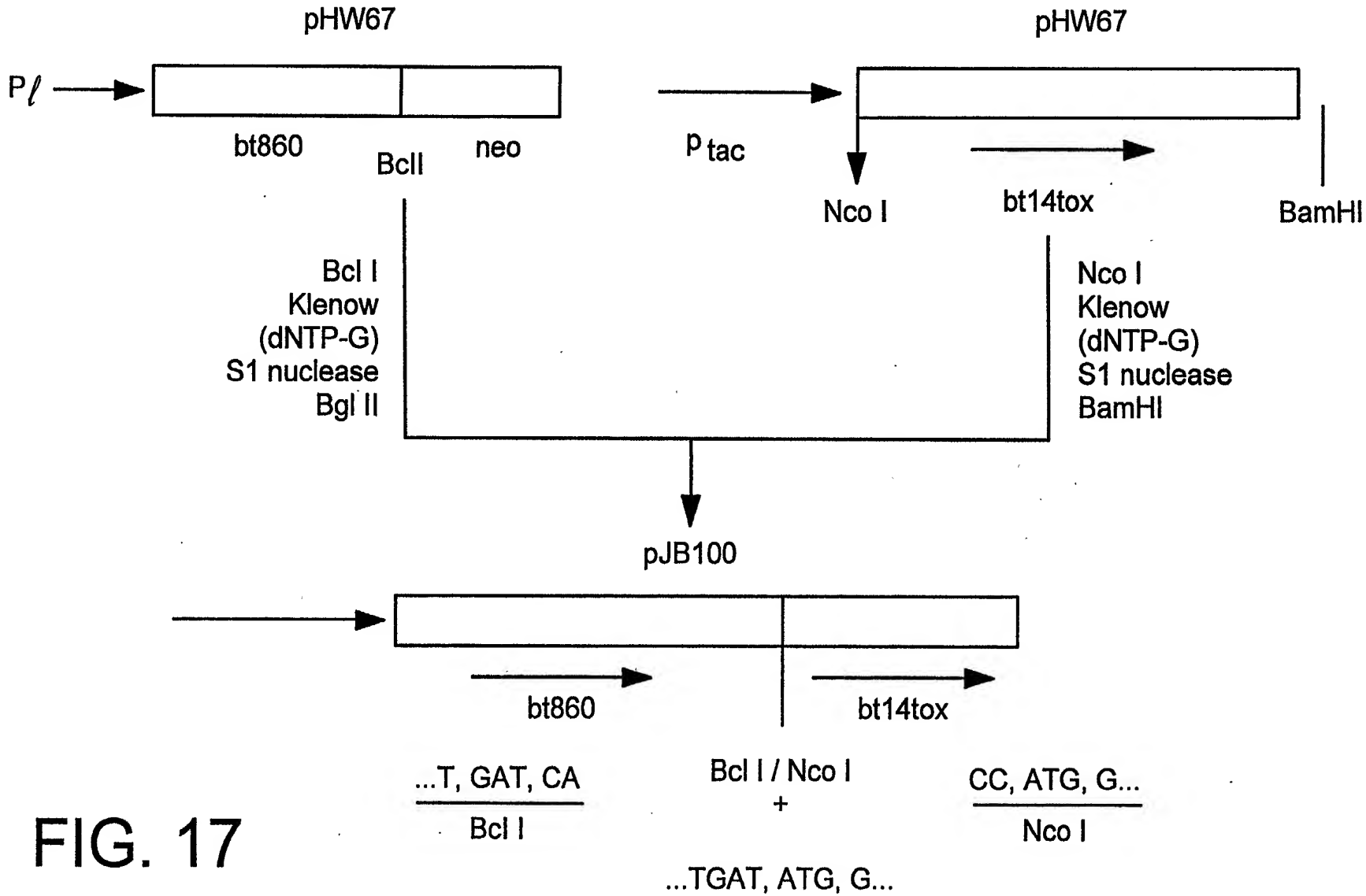


FIG. 17

Declaration, Power Of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PREVENTION OF Bt RESISTANCE DEVELOPMENT

the specification of which

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____.

☒ was filed as PCT international application

Number PCT/EP 90/00905

on May 30, 1990,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information material to the examination of this application in accordance with Section 1.56(a) of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under Section 119 of Title 35 United States Code, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Day/Month/Year	Priority Claimed
89401499.2	UNITED KINGDOM	31/05/1989	☒ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, We (I) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
PCT/EP 90/00905	*MAY 30, 1990*	

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

William L. Mathis	17,337	Norman H. Stepno	22,716	Robert G Mukai	28,531
Peter H. Smolka	15,913	Ronald L. Grudziecki	24,970	George A Hovanec, Jr	28,223
Robert S. Swecker	19,885	Frederick G Michaud, Jr.	26,003	James A. LaBarre	28,632
Platon N Mandros	22,124	Alan E Kopecki	25,813	E. Joseph Gess	28,510
Benton S Duffett, Jr	22,030	Regis E. Slutter	26,999	David D. Reynolds	29,273
Joseph R Magnone	24,239	Samuel C Miller, III	27,360	R. Danny Huntington	27,903
Joel M. Freed	25,101	Ralph L. Freeland, Jr.	16,110		

and: _____

Address all correspondence to:

Burns, Doane, Swecker & Mathis
George Mason Building
Washington and Prince Streets
P. O. Box 1404
Alexandria, Virginia 22313-1404

Address all telephone calls to: _____ at (703) 836-6620.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

VAN MELLAERT Herman
NAME OF FIRST SOLE INVENTOR

Signature of Inventor

26/11/90
Date

Residence: Wilselsesteénweg 19
B-3200 LEUVEN (Belgium)

Citizenship: Belgian

Post Office Address: the same

DOCKETED
filed 10/22/90

BOTTERMAN Johan

NAME OF SECOND JOINT INVENTOR

Signature of Inventor

26/11/1990.

Date

Residence: Het Wijngaardeke 5
B ~~9721~~ 9840 ZEVERGEM-DE PINTE (Belgium)

Citizenship: Belgian

Post Office Address: the same

VAN RIE Jeroen

NAME OF THIRD JOINT INVENTOR

Signature of Inventor

Nov. 26, 1990.

Date

Residence: Gravin Johannalaan 10
B 9900 EEKLO (Belgium)

Citizenship: ~~Belgian~~

Post Office Address: the same

JOOS Henk

NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

26 November 1990

Date

Residence: Oostmolen Zuid 5
~~B-9880 AALTER (Belgium)~~

Citizenship: Belgian

Post Office Address: ~~the same~~

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

Residence:

Citizenship:

Post Office Address:

Patent
Attorney's Docket No. 021565-078

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Herman VAN MELLAERT et al.	)	Group Art Unit: Unassigned
	)	
Application No.: TBA (Div of 09/176,320)	)	Examiner: Unassigned
	)	
Filed: Even date herewith	)	
	)	
For: RECOMBINANT PLANT EXPRESSING NON-COMPETITIVELY BINDING Bt INSECTICIDAL CRYSTAL PROTEINS	)	

REQUEST FOR TRANSFER OF COMPUTER READABLE SEQUENCE LISTING FROM ANOTHER APPLICATION TO THE PRESENT APPLICATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The computer readable form in this application, TBA (Div of 09/176,320), is identical with that filed in Application Serial No. 09/176,320, filed October 19, 1998. In accordance with 37 C.F.R. §1.821(e), please use the only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is included in a separately filed preliminary amendment for incorporation into the specification.

Applicants' undersigned representative hereby affirms:

1. That the content of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. 1.821(c) and (e), respectively, are the same in compliance with 1.821(f); and

2. That the submission, filed in accordance with 37 C.F.R. 1.821(g) herein does not include new matter.

In the event that there are any questions relating to this request, or to the application in general, it would be appreciated if the Examiner would telephone the undersigned attorney concerning such questions so that prosecution of this application may be expedited.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§1.116 and 1.117 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: Malcolm K. McGowan

Malcolm K. McGowan, Ph.D.
Registration No. 39,300

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

Date: 13 September 2000

SEQUENCE LISTING

<110> Van Mellaert, Herman

Botterman, Johan

Van Rie, Jeroen

Joos, Henk

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cagtatcaaa ctatcagaac tttggtagtt taataaaaaa acggaggtat ttt atg 236

Met

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gag gaa aat aat caa aat caa tgc ata cct tac aat tgt tta agt aat 284

Glu Glu Asn Asn Gln Asn Gln Cys Ile Pro Tyr Asn Cys Leu Ser Asn

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Pro Glu Glu Val Leu Leu Asp Gly Glu Arg Ile Ser Thr Gly Asn Ser

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Ser Ile Asp Ile Ser Leu Ser Leu Val Gln Phe Met Val Ser Asn Phe

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Val Pro Gly Gly Gly Phe Leu Val Gly Leu Ile Asp Phe Val Trp Gly

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ata gtt ggc cct tct caa tgg gat gca ttt cta gta caa att gaa caa 476

Ile Val Gly Pro Ser Gln Trp Asp Ala Phe Leu Val Gln Ile Glu Gln

70 75 80

tta att aat gaa aga ata gct gaa ttt gct agg aat gct gct att gct 524

Leu Ile Asn Glu Arg Ile Ala Glu Phe Ala Arg Asn Ala Ala Ile Ala

85 90 95

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Asn Leu Glu Gly Leu Glu Asn Asn Leu Asn Ile Tyr Val Glu Ala Phe

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aaa gaa tgg gaa gaa gat cct aat aat cca gaa acc agg acc aga gta 620

Lys Glu Trp Glu Glu Asp Pro Asn Asn Pro Glu Thr Arg Thr Arg Val

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Ile Asp Arg Phe Arg Ile Leu Asp Gly Leu Leu Glu Arg Asp Ile Pro

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tcg ttt cga att tct gga ttt gaa gta ccc ctt tta tcc gtt tat gct 716

Ser Phe Arg Ile Ser Gly Phe Glu Val Pro Leu Leu Ser Val Tyr Ala

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caa gcg gcc aat ctg cat cta gct ata tta aga gat tct gta att ttt 764

Gln Ala Ala Asn Leu His Leu Ala Ile Leu Arg Asp Ser Val Ile Phe

165 170 175

gga gaa aga tgg gga ttg aca acg ata aat gtc aat gaa aac tat aat 812
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aga cta att agg cat att gat gaa tat gct gat cac tgt gca aat acg 860
Arg Leu Ile Arg His Ile Asp Glu Tyr Ala Asp His Cys Ala Asn Thr
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Tyr Asn Arg Gly Leu Asn Asn Leu Pro Lys Ser Thr Tyr Gln Asp Trp
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Ile Thr Tyr Asn Arg Leu Arg Arg Asp Leu Thr Leu Thr Val Leu Asp
230 235 240

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Ile Ala Ala Phe Phe Pro Asn Tyr Asp Asn Arg Arg Tyr Pro Ile Gln
245 250 255

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Pro Val Gly Gln Leu Thr Arg Glu Val Tyr Thr Asp Pro Leu Ile Asn

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Met Glu Ser Ser Ala Ile Arg Asn Pro His Leu Phe Asp Ile Leu Asn

290 295 300 305

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Asn Leu Thr Ile Phe Thr Asp Trp Phe Ser Val Gly Arg Asn Phe Tyr

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Trp Gly Gly His Arg Val Ile Ser Ser Leu Ile Gly Gly Gly Asn Ile

325 330 335

aca tct cct ata tat gga aga gag gcg aac cag gag cct cca aga tcc 1292

Thr Ser Pro Ile Tyr Gly Arg Glu Ala Asn Gln Glu Pro Pro Arg Ser

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Phe Thr Phe Asn Gly Pro Val Phe Arg Thr Leu Ser Asn Pro Thr Leu

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Arg Leu Leu Gln Gln Pro Trp Pro Ala Pro Pro Phe Asn Leu Arg Gly

370 375 380 385

gtt gaa gga gta gaa ttt tct aca cct aca aat agc ttt acg tat cga 1436

Val Glu Gly Val Glu Phe Ser Thr Pro Thr Asn Ser Phe Thr Tyr Arg

390 395 400

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Gly Arg Gly Thr Val Asp Ser Leu Thr Glu Leu Pro Pro Glu Asp Asn

405 410 415

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Ser Val Pro Pro Arg Glu Gly Tyr Ser His Arg Leu Cys His Ala Thr

420 425 430

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Phe Val Gln Arg Ser Gly Thr Pro Phe Leu Thr Thr Gly Val Val Phe

435 440 445

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Ser Trp Thr His Arg Ser Ala Thr Leu Thr Asn Thr Ile Asp Pro Glu

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aga att aat caa ata cct tta gtg aaa gga ttt aga gtt tgg ggg ggc 1676

Arg Ile Asn Gln Ile Pro Leu Val Lys Gly Phe Arg Val Trp Gly Gly

470 475 480

acc tct gtc att aca gga cca gga ttt aca gga ggg gat atc ctt cga 1724

Thr Ser Val Ile Thr Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Arg

485 490 495

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Arg Asn Thr Phe Gly Asp Phe Val Ser Leu Gln Val Asn Ile Asn Ser

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595 600 605

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Ile Glu Ile Ile Leu Ala Asp Ala Thr Phe Glu Ala Glu Ser Asp Leu
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Glu Arg Ala Gln Lys Ala Val Asn Ala Leu Phe Thr Ser Ser Asn Gln

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660 665 670

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675 680 685

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740 745 750

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Tyr Thr Arg Tyr Glu Leu Arg Gly Tyr Ile Glu Asp Ser Gln Asp Leu

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790 795 800

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Lys Cys Gly Glu Pro Asn Arg Cys Ala Pro His Leu Glu Trp Asn Pro

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Asp Leu Asp Cys Ser Cys Arg Asp Gly Glu Lys Cys Ala His His Ser

820 825 830

cat cat ttc acc ttg gat att gat gtt gga tgt aca gac tta aat gag 2780

His His Phe Thr Leu Asp Ile Asp Val Gly Cys Thr Asp Leu Asn Glu

835 840 845

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870 875 880

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885 890 895

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1125 1130 1135

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1140 1145 1150

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1155 1160 1165

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Ser Ser Ile Asp Ile Ser Leu Ser Leu Val Gln Phe Met Val Ser Asn

35 40 45

Phe Val Pro Gly Gly Gly Phe Leu Val Gly Leu Ile Asp Phe Val Trp

50 55 60

Gly Ile Val Gly Pro Ser Gln Trp Asp Ala Phe Leu Val Gln Ile Glu

65 70 75 80

Gln Leu Ile Asn Glu Arg Ile Ala Glu Phe Ala Arg Asn Ala Ala Ile

85 90 95

Ala Asn Leu Glu Gly Leu Glu Asn Asn Leu Asn Ile Tyr Val Glu Ala

100 105 110

Phe Lys Glu Trp Glu Glu Asp Pro Asn Asn Pro Glu Thr Arg Thr Arg

115 120 125

Val Ile Asp Arg Phe Arg Ile Leu Asp Gly Leu Leu Glu Arg Asp Ile

130 135 140

Pro Ser Phe Arg Ile Ser Gly Phe Glu Val Pro Leu Leu Ser Val Tyr

145 150 155 160

Ala Gln Ala Ala Asn Leu His Leu Ala Ile Leu Arg Asp Ser Val Ile

165 170 175

Phe Gly Glu Arg Trp Gly Leu Thr Thr Ile Asn Val Asn Glu Asn Tyr

180 185 190

Asn Arg Leu Ile Arg His Ile Asp Glu Tyr Ala Asp His Cys Ala Asn

195 200 205

Thr Tyr Asn Arg Gly Leu Asn Asn Leu Pro Lys Ser Thr Tyr Gln Asp

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Trp Ile Thr Tyr Asn Arg Leu Arg Arg Asp Leu Thr Leu Thr Val Leu

225 230 235 240

Asp Ile Ala Ala Phe Phe Pro Asn Tyr Asp Asn Arg Arg Tyr Pro Ile

245 250 255

Gln Pro Val Gly Gln Leu Thr Arg Glu Val Tyr Thr Asp Pro Leu Ile

260 265 270

Asn Phe Asn Pro Gln Leu Gln Ser Val Ala Gln Leu Pro Thr Phe Asn

275 280 285

Val Met Glu Ser Ser Ala Ile Arg Asn Pro His Leu Phe Asp Ile Leu

290 295 300

Asn Asn Leu Thr Ile Phe Thr Asp Trp Phe Ser Val Gly Arg Asn Phe

305 310 315 320

Tyr Trp Gly Gly His Arg Val Ile Ser Ser Leu Ile Gly Gly Gly Asn

325 330 335

Ile Thr Ser Pro Ile Tyr Gly Arg Glu Ala Asn Gln Glu Pro Pro Arg

340 345 350

Ser Phe Thr Phe Asn Gly Pro Val Phe Arg Thr Leu Ser Asn Pro Thr

355 360 365

Leu Arg Leu Leu Gln Gln Pro Trp Pro Ala Pro Pro Phe Asn Leu Arg

370 375 380

Gly Val Glu Gly Val Glu Phe Ser Thr Pro Thr Asn Ser Phe Thr Tyr

385 390 395 400

Arg Gly Arg Gly Thr Val Asp Ser Leu Thr Glu Leu Pro Pro Glu Asp

405 410 415

Asn Ser Val Pro Pro Arg Glu Gly Tyr Ser His Arg Leu Cys His Ala

420 425 430

Thr Phe Val Gln Arg Ser Gly Thr Pro Phe Leu Thr Thr Gly Val Val

435 440 445

Phe Ser Trp Thr His Arg Ser Ala Thr Leu Thr Asn Thr Ile Asp Pro

450 455 460

Glu Arg Ile Asn Gln Ile Pro Leu Val Lys Gly Phe Arg Val Trp Gly

465 470 475 480

Gly Thr Ser Val Ile Thr Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu

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Arg Arg Asn Thr Phe Gly Asp Phe Val Ser Leu Gln Val Asn Ile Asn

500 505 510

Ser Pro Ile Thr Gln Arg Tyr Arg Leu Arg Phe Arg Tyr Ala Ser Ser

515 520 525

Arg Asp Ala Arg Val Ile Val Leu Thr Gly Ala Ala Ser Thr Gly Val

530 535 540

Gly Gly Gln Val Ser Val Asn Met Pro Leu Gln Lys Thr Met Glu Ile

545 550 555 560

Gly Glu Asn Leu Thr Ser Arg Thr Phe Arg Tyr Thr Asp Phe Ser Asn

565 570 575

Pro Phe Ser Phe Arg Ala Asn Pro Asp Ile Ile Gly Ile Ser Glu Gln

580 585 590

Pro Leu Phe Gly Ala Gly Ser Ile Ser Ser Gly Glu Leu Tyr Ile Asp

595 600 605

Lys Ile Glu Ile Ile Leu Ala Asp Ala Thr Phe Glu Ala Glu Ser Asp

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Leu Glu Arg Ala Gln Lys Ala Val Asn Ala Leu Phe Thr Ser Ser Asn

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Gln Ile Gly Leu Lys Thr Asp Val Thr Asp Tyr His Ile Asp Gln Val

645 650 655

Ser Asn Leu Val Asp Cys Leu Ser Asp Glu Phe Cys Leu Asp Glu Lys

660 665 670

Arg Glu Leu Ser Glu Lys Val Lys His Ala Lys Arg Leu Ser Asp Glu

675 680 685

Arg Asn Leu Leu Gln Asp Pro Asn Phe Arg Gly Ile Asn Arg Gln Pro

690 695 700

Asp Arg Gly Trp Arg Gly Ser Thr Asp Ile Thr Ile Gln Gly Gly Asp

705 710 715 720

Asp Val Phe Lys Glu Asn Tyr Val Thr Leu Pro Gly Thr Val Asp Glu

725 730 735

Cys Tyr Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu Ser Lys Leu Lys

740 745 750

Ala Tyr Thr Arg Tyr Glu Leu Arg Gly Tyr Ile Glu Asp Ser Gln Asp

755 760 765

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770 775 780

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785 790 795 800

Gly Lys Cys Gly Glu Pro Asn Arg Cys Ala Pro His Leu Glu Trp Asn

805 810 815

Pro Asp Leu Asp Cys Ser Cys Arg Asp Gly Glu Lys Cys Ala His His

820 825 830

Ser His His Phe Thr Leu Asp Ile Asp Val Gly Cys Thr Asp Leu Asn

835 840 845

Glu Asp Leu Gly Val Trp Val Ile Phe Lys Ile Lys Thr Gln Asp Gly

850 855 860

His Ala Arg Leu Gly Asn Leu Glu Phe Leu Glu Glu Lys Pro Leu Leu

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Gly Glu Ala Leu Ala Arg Val Lys Arg Ala Glu Lys Lys Trp Arg Asp

885 890 895

Lys Arg Glu Lys Leu Gln Leu Glu Thr Asn Ile Val Tyr Lys Glu Ala

900 905 910

Lys Glu Ser Val Asp Ala Leu Phe Val Asn Ser Gln Tyr Asp Arg Leu

915 920 925

Gln Val Asp Thr Asn Ile Ala Met Ile His Ala Ala Asp Lys Arg Val

930 935 940

His Arg Ile Arg Glu Ala Tyr Leu Pro Glu Leu Ser Val Ile Pro Gly

945 950 955 960

Val Asn Ala Ala Ile Phe Glu Glu Leu Glu Gly Arg Ile Phe Thr Ala

965 970 975

Tyr Ser Leu Tyr Asp Ala Arg Asn Val Ile Lys Asn Gly Asp Phe Asn

980 985 990

Asn Gly Leu Leu Cys Trp Asn Val Lys Gly His Val Asp Val Glu Glu

995 1000 1005

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1075 1080 1085

Thr Gln Glu Glu Tyr Glu Gly Thr Tyr Thr Ser Arg Asn Gln Gly Tyr

1090 1095 1100

Asp Glu Ala Tyr Gly Asn Asn Pro Ser Val Pro Ala Asp Tyr Ala Ser

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Val Tyr Glu Glu Lys Ser Tyr Thr Asp Gly Arg Arg Glu Asn Pro Cys

1125 1130 1135

Glu Ser Asn Arg Gly Tyr Gly Asp Tyr Thr Pro Leu Pro Ala Gly Tyr

1140 1145 1150

Val Thr Lys Asp Leu Glu Tyr Phe Pro Glu Thr Asp Lys Val Trp Ile

1155 1160 1165

Glu Ile Gly Glu Thr Glu Gly Thr Phe Ile Val Asp Ser Val Glu Leu

1170 1175 1180

Leu Leu Met Glu Glu

185